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SERUM DIAGNOSIS BY COMPLEMENT-FIXATION

WITH SPECIAL REFERENCE TO SYPHILIS

THE PRINCIPLES, TECHNIQUE AND CLINICAL APPLICATIONS

BY

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JOHN H. STOKES, M.D.

MASTER SYPHILOLOGIST

AND

MY ASSISTANTS

WHOSE NAMES APPEAR SO FREQUENTLY IN THE FOLLOWING PAGES

AND

IN MEMORY

OF

ALLEN J. SMITH, M.D., Sc.D., LL.D.

FORMERLY PROFESSOR OF PATHOLOGY IN THE UNDERGRADUATE SCHOOL OF MEDICINE OF THE UNIVERSITY OF PENNSYLVANIA

BELOVED TEACHER, FRIEND AND COLLEAGUE

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PREFACE.

This book is essentially a summary of the clinical and laboratory investigations which I have made during the past nine years in serum diagnosis by complement-fixation, not only in syphilis but likewise in the wide field of the bacterial, protozoal and metazoal diseases of human beings and the lower animals and in the identification of blood and seminal stains, the detection of meat and milk adulterations and other soluble albumins. The results of studies bearing upon the latter subjects are here published for the first time in accordance with the advice of numerous friends who thought this plan preferable to the publication of separate papers.

The book does not aim to be a complete review or compilation of the enormous literature upon the Wassermann reaction, excellent reviews being found in the works of Browning and McKenzie, Bruck, Craig, Noguchi and others, or of the literature upon complement-fixation in other diseases, but is mostly an account of original investigations and the description of new methods along with practical applications, including numerous references to the investigations of others with them. It has been prepared not only for serologists and laboratory technicians but likewise and especially for practicing physicians and veterinarians.

In Part I I have sought to state briefly and clearly the important underlying principles of serum hemolysis and complement-fixation since a good working knowledge of them is *essential* for the proper conduct of the tests by serologists and laboratory technicians and helpful to clinicians for the proper interpretation of reactions.

Part II is devoted to the subject of the principles of technique so important in the conduct of tests aiming to possess the maximum of sensitiveness consistent with specificity. So much harm has been done by improperly qualified workers as well as by defective methods especially in the Wassermann reaction, that I have sought to discover the sources of error and to prevent them in the methods proposed.

I fully realize that no amount of investigation and no test however simple can prevent technical errors due to the personal equation of individual workers, but in Part III I have aimed to present the technique of my methods simply and in *detail* hoping that those who adopt them will follow the directions *exactly* as described. In

some laboratories my new Wassermann test has been so greatly modified that it scarcely bears a resemblance to my technique. Furthermore, the new antigen is sometimes used in other methods which may give the impression that the test itself is being employed whereas it was designed and meant only for use in the new method.

Part IV is devoted to an account of the sensitiveness, specificity and practical applications of the methods in the serum diagnosis and treatment of syphilis, tuberculosis, gonorrhea, glanders, contagious abortion, typhoid fever, echinococcus disease, dourine and numerous other bacterial, protozoal and metazoal diseases of human beings and the lower animals as well as for the medico-legal identification of blood and seminal stains, the detection of meat and milk adulterations, identification of bacteria, etc. Special emphasis is placed upon the results observed by others and myself with my new antigen and method in the diagnosis of syphilis and as a serological guide to its treatment, these having become widely adopted since described in 1922 and a large amount of clinical and laboratory data accumulated. In preparing this section I have also drawn upon my own clinical experience in the diagnosis and treatment of syphilis and I am confident that others will observe the same results if the tests are conducted as described, which is exactly the way they are conducted in my laboratory.

In conclusion I beg to express my indebtedness to Miss E. M. Yagle and Miss C. Richter for assistance in reading the proofs, to Mr. Herman Brown for several of the illustrations and to Dr. Fred Boerner for assistance in preparing the chapter devoted to complement-fixation in diseases of the lower animals; also my deep appreciation of the unvarying efficiency and courtesy of the publishers.

J. A. K.

PHILADELPHIA, PA.

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Sensitiveness, Specificity and Practical Value of the Kolmer Complement-fixation Reaction for the Detection of Meat Adulteration.

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CHAPTER LIII.

Sensitiveness, Specificity and Practical Value of the Kolmer Complement-fixation Reaction for the Detection of Milk Adulteration.

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SERUM DIAGNOSIS BY COMPLEMENT-FIXATION.

PART I.

PRINCIPLES OF SERUM HEMOLYSIS AND COMPLEMENT-FIXATION.

CHAPTER I.

HISTORY OF SERUM HEMOLYSIS AND COMPLEMENT-FIXATION.

The phenomenon of serum hemolysis in the test-tube was first studied and clearly defined by Bordet[1] in 1898. It is true that Landois in 1876 had described the hemolytic activity of fresh human serum for the erythrocytes of some of the lower animals in relation to the accidents of blood transfusion and Buchner about fifteen years later had again drawn attention to the fact that the fresh serum of one animal may hemolyze the corpuscles of an unrelated animal, but Bordet was first to show that the phenomenon was similar to bacteriolysis and that when the corpuscles of the rabbit were injected repeatedly into the peritoneal cavity of the guinea-pig, the serum of the latter acquired the property of agglutinating and hemolyzing rabbit erythrocytes in the test-tube. In other words, Landois and Buchner had previously observed the normal or natural hemolysins of serum while Bordet was first to describe the immune hemolysins and to show that two substances were concerned in the phenomenon, namely, the specific antibody or "substance sensibilisatrice" and the "alexin" or non-specific natural constituent of serum since renamed and better known as complement.

Naturally with a disease so widespread and important as syphilis many attempts were made to establish a method for its laboratory diagnosis by various hemolysin, hemagglutination and precipitin tests prior to the discovery of *Spirochæta pallida* in 1905. But none of these commanded much attention and almost the same may be

stated for the first complement-fixation test conducted by Bordet and Gengou[2] in 1901, to settle an argument with Ehrlich on the question of the unity or multiplicity of complement.

Bordet, who, as just mentioned, had previously discovered that serum hemolysis was due to an hemolysin or hemolytic amboceptor specific or homologous for the corpuscles employed and a second non-specific constituent of serum designated as alexin or complement expressed his belief in the existence of one complement capable of acting with or being "fixed" by either a hemolytic or bacteriolytic amboceptor; Ehrlich and Morgenroth soon confirmed these discoveries but maintained that any normal serum may contain several complements. To prove his contention Bordet, with the assistance of Gengou, placed in test-tubes 0.4 cc. of a suspension of *B. pestis*, added 1.2 cc. of heated antipest serum containing specific amboceptors for the antigen (*B. pestis*) and 0.2 cc. of guinea-pig serum to furnish complement. Duplicate tests were set up using normal instead of antipest serum. After an interval of several hours at room temperature they added to each tube 2 drops of a suspension of washed rabbit corpuscles sensitized with antirabbit hemolysin and after a secondary incubation found that the tubes carrying *B. pestis*, antipest serum and complement gave no hemolysis because of a previous fixation of complement whereas hemolysis occurred in those tubes carrying *B. pestis* and normal horse serum because complement had not been fixed. Additional controls on the antigen and sera alone were included; indeed the whole experiment was wonderfully correct in technique and the results were accepted by Bordet as proving the unity of complement of guinea-pig serum since it was fixable by either bacteriolytic or hemolytic amboceptors. Additional tests employing antigens of *B. typhosus* and *B. vulgaris* and their immune sera yielded similar results. At the same time Bordet also showed that sensitized cholera vibrios added to guinea-pig serum underwent granulation and lysis, this phenomenon of bacteriolysis exhausting the alexin or complement as shown by the fact that sensitized rabbit erythrocytes subsequently added did not undergo hemolysis. Or if the process was reversed and sensitized corpuscles first added to guinea-pig serum complement, it was found that the resulting hemolysis exhausted the complement and left none to produce lysis of sensitized vibrios; therefore, Bordet argued, there is but one complement capable of acting with either bacteriolytic or hemolytic sensitizers.

Thus was conducted the first complement-fixation test as the term is understood at present. Really, however, the discovery dates back to 1898 when Bordet unraveled the mechanism of serum hemolysis, because when corpuscles, specific antibody, and complement are brought together in a test-tube in proper proportions, the antibody unites with the corpuscles and this aggregate absorbs

or fixes the complement. This is as pure an example of specific complement-fixation as the fixation of complement by antipest serum and an antigen of *B. pestis*. But curiously enough the practical applications entirely escaped Bordet, Gengou, Ehrlich and Morgenroth although quickly and first foreseen by Widal and Lesourd[3] in 1901, who applied the Bordet-Gengou phenomenon of complement-fixation to the serum diagnosis of typhoid fever and tuberculosis, but with indifferent success. However, these investigators found that positive reactions sometimes occurred earlier than positive agglutination reactions, when employing an antigen of *B. typhosus* and the sera of typhoid fever patients; since then their observations have been amply confirmed and, as stated in Chapter XLV, it is to be regretted that the superior practical application of complement-fixation in the serum diagnosis of typhoid fever has been so long over-shadowed by the Widal agglutination test.

The same may be stated of complement-fixation tests for the identification and differentiation of such substances as serum, blood, milk, etc., since Gengou[4] in 1902 and Neisser and Sachs[5] in 1905 showed that the complement-fixation reaction was much more sensitive than the precipitin test for the detection and differentiation of soluble albuminous substances, a subject which I shall likewise discuss with more detail in a later chapter and particularly in the application of the complement-fixation test for the medico-legal detection and differentiation of blood, semen, etc.

Encouraged by these results Wassermann and Bruck studied complement-fixation in typhoid fever and meningococcus meningitis, their study being followed by Citron's work on swine erysipelas, Weil on cholera, Müller and Oppenheim on gonorrhea, etc. Wassermann and Sachs endeavored to demonstrate the existence of antigens in the tissues by means of the complement-fixation test and for this purpose employed extracts of tuberculous glands and tuberculin as antigens in tests with antituberculosis and human tuberculosis sera. At this time Schaudinn made his great discovery of *Spirochæta pallida* which naturally focussed the attention of the medical world upon syphilis. This was quickly followed by Neisser's work on experimental syphilis of monkeys which led to the application of the complement-fixation reaction in the serum diagnosis of this disease. However, I am unable to state who first suggested this epoch-making investigation. Bruck[6] claims that while Wassermann first suggested the complement-fixation test for demonstrating spirochetal antigen in saline extracts of syphilitic tissues, he was first to develop the idea experimentally and to show Wassermann the behavior of syphilitic serum in their presence; Wassermann[7] states that the first suggestion for applying the complement-fixation test in syphilis of the monkey was made by Althoff. Be that as it may, Wasser-

mann, Neisser and Bruck[8] were first to publish the results of their work upon complement-fixation in syphilis of monkeys followed by that of Wassermann, Neisser, Bruck and Schucht[9] upon the results of a large number of complement-fixation tests in syphilis in Neisser's clinic. Since then the test has become the best and most widely known of all laboratory methods under the phrase "Wassermann reaction."

But just two weeks later Detre[10] published a paper recording his independent work on the same subject and I have always believed him a worthy sharer of the honors. Using aqueous extracts of syphilitic papules, liver, pancreas and tonsillar exudates as antigens, he performed complement-fixation tests with an antihorse hemolytic system and the sera of 6 syphilitic and 4 normal individuals, finding positive reactions with 2 of the 6 syphilitics. A little later Wassermann and Plaut[11] reported that in tests applied to the cerebrospinal fluids of 41 cases of paresis, positive reactions occurred in 32; this, I believe, was the first application of complement-fixation employing spinal fluid instead of serum and in 1907 it was followed by Levaditi's and Marie's[12] report upon positive complement-fixation reactions with the spinal fluid in tabes dorsalis. Naturally these results greatly stimulated the application of the complement-fixation test to other diseases and in 1905 and 1906 we find not only the reports of Müller and Oppenheim bearing upon complement-fixation in gonorrhea but likewise those of Wassermann and Bruck upon tuberculosis, Bordet and Gengou upon pertussis, Ghedini upon echinococcus disease, followed in later years by numerous reports relating to the successful application of the test in the serum diagnosis of dourine, glanders, contagious abortion of cattle, etc. Among these early investigators were also Gay[13] and Moreschi[14] who applied themselves more particularly to a study of complement-fixation by precipitates, as a means for explaining the phenomenon of anticomplementary action of sera, which we now realize is of fundamental importance in the mechanism of the complement-fixation reaction.

Up to this time the syphilitic reaction was regarded as but a simple and direct application of Bordet's and Gengou's phenomenon of specific complement-fixation, requiring a specific syphilitic antigen before complement could be fixed by syphilis antibody. But in 1907, Weygandt[15] first showed that the Wassermann reaction was not biologically specific since positive reactions were found to occur with aqueous extracts of normal guinea-pig spleen. This work was soon confirmed, especially by Landsteiner, Müller and Pötzl,[16] who found that positive reactions occurred with alcoholic extracts of guinea-pig heart. They showed that the antigenic principle was lipoidal, soluble in alcohol, and initiated a host of studies upon other lipoidal substances like sodium taurocholate and glycocholate, cholesterol, oleic acid, etc., as so-called "antigens" for the syphilis reaction.

These discoveries on the biologically non-specific character of the Wassermann reaction along with the widespread use of the test by inexperienced individuals, as well as the many sources of technical error, tended to retard an earlier appreciation of its great practical value, and served to swing the pendulum of medical opinion so far in the wrong direction that the effects are yet to be dealt with. Due almost entirely to technical errors, falsely positive reactions have since been reported by some one at some time in almost all the diseases liable to afflict humanity. The careful work of Citron, however, was largely instrumental in preserving the importance of the reaction until a better understanding of the technique resulted in improved and more careful work, with a greater respect for the real value of the complement-fixation reaction in the serum diagnosis of not only syphilis but of various bacterial diseases and for the identification and differentiation of proteins. Indeed, as so well stated by Vedder "one is impressed by the fact that the Wassermann reaction must be a test of surprising merit to have survived all the clumsy technique that has been perpetrated in its name."

But regarding the use of the phrase "Wassermann reaction," I believe[17] that it is employed altogether too freely and loosely and has really come into use as a synonym for *complement-fixation* in syphilis. It is rather carelessly applied by many persons to any complement-fixation test for syphilis regardless of how remote the method may be from Wassermann's test described in 1906; Vedder[18] believes that the term should be discontinued altogether. Strictly speaking it should be confined to a test conducted with Wassermann's original technique, but practically, the latitude may be broader and embrace those modifications employing alcoholic extracts of syphilitic and non-syphilitic tissues as "antigens" and such changes as using each constituent of the test in one-half, one-quarter or one-tenth the amounts of the original Wassermann test inasmuch as these changes do not alter the principles and are used mainly for economy.

However, all tests employing a different method for adjusting the hemolytic system, a method for titrating complement, a different hemolytic system, the use of active instead of heated serum, the utilization of natural complements and amboceptors in patients' sera, etc., cannot claim the designation "Wassermann test," being based upon principles and technique too remote from Wassermann's technique. And the literature is full of these various modifications embracing the principles of the complement-fixation reaction but otherwise radically different from the original Wassermann test and adding greatly to the confusion of the subject. Some of the best known of these modifications are those of Noguchi employing an antihuman hemolytic system, the Hecht-Weinberg employing the natural complement and antisheep hemolysin of human sera,

and numerous modifications of these tests striving to simplify the technique and increase the sensitiveness of the reactions.

Finally it would appear advisable to exercise more care in the use of the terms "reaction" and "test." In common with the majority of persons, I have used the terms as synonymous but believe that the term "test" should be used in designating the *technique or method* as "a means of trial," and "reaction" for expressing the *results* of the test; for example, a serum is submitted to a laboratory for the "complement-fixation test" and the results designated as a positive or negative "reaction."

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CHAPTER II.

NATURE AND MECHANISM OF SERUM HEMOLYSIS.

Hemolysis means the solution or lysis of erythrocytes with liberation of hemoglobin. As would be expected a large number of agents are capable of producing hemolysis because of the delicate structure of these cells. But only a few of these are of interest and importance in relation to the complement-fixation test, namely, hemolysis produced by the (1) mechanical injury of corpuscles, as may occur when blood is defibrinated; (2) by temperature changes, as temperatures above 50° C. or alternate freezing and thawing; (3) by water, hypotonic or hypertonic salt solutions used in preparing suspensions of erythrocytes and dilutions of various other reagents; (4) by acids, alkalies or other chemical substances in relation to the glassware and saline solution employed in serum hemolysis and complement-fixation tests; (5) by tissue extracts and especially their lipoidal constituents, in relation to extracts employed as "antigen" for the syphilis complement-fixation test; (6) by bacterial and other vegetable toxins in relation to the preparation of antigens for complement-fixation tests employing them, and finally (7) by antibody in the serum called hemolysin, sensitizer or hemolytic amboceptor which, acting in conjunction with complement, produces serum hemolysis. All except the last are examples of *non-specific hemolysis* because they are capable of hemolyzing the erythrocytes of any animal. The last or serum hemolysis however, is an example of *specific hemolysis* because the antibody will sensitize or prepare only the erythrocytes of that species for which it possesses a specific affinity. All of the non-specific hemolytic agents mentioned will be discussed at greater length in succeeding chapters according as they come into relation with the technique of complement-fixation tests. The balance of the present chapter will be devoted to the subject of the nature and mechanism of serum hemolysis.

The phenomenon of serum hemolysis is particularly of interest and importance in relation to serum diagnosis by complement-fixation not only because it is in itself an example of complement-fixation but likewise because it plays an essential part in every complement-fixation test applied in the diagnosis of disease. Since described by Bordet in 1898, a great deal of investigation has been devoted to elucidating its nature and mechanism, and while it cannot be stated that these problems have been entirely and suc-

cessfully solved, yet a large amount of accurate information has been gained of direct application to the complement-fixation phenomenon in serum diagnosis.

PRINCIPLES OF SERUM HEMOLYSIS.

Nomenclature.—*Serum hemolysis* has been so designated because the two substances bringing it about are to be found in the plasma or serum of the blood. One of these substances is of the nature of a specific antibody which unites with the erythrocytes and prepares or sensitizes them to the dissolving or lytic effect of the second non-specific substance. The former or specific antibody was called "substance sensibilisatrice" by Bordet but subsequently renamed "hemolytic amboceptor" by Ehrlich. Since then it has been called "sensitizer," "amboceptor" and "hemolysin;" my preference is for the latter term.

The second or non-specific substance was called "alexin" by Bordet and "complement" by Ehrlich. The latter however, has come into more general use and is to be preferred to the former because the term "alexin" was first coined by Buchner to designate the total bacteriolytic and hemolytic activity of serum which we now know is due not only to this non-specific substance but likewise to the specific antibody.

Serum hemolysis is due therefore, to the interaction of three substances, namely, the erythrocytes sensitized or prepared by their specific antibody called the hemolytic amboceptor, sensitizer or hemolysin; this is followed by an actual destruction of the corpuscles by the second or non-specific constituent of serum designated as complement. The antibody alone is unable to hemolyze erythrocytes. The complement alone is likewise unable to hemolyze; hemolysis is caused by the combined action of the two substances.

The antibody or hemolysin is designated according to the kind of corpuscles for which it has an affinity. For example, the hemolysin for sheep corpuscles is called antisheep hemolysin, sensitizer or amboceptor; that for human erythrocytes is known as antihuman hemolysin, etc. Corpuscles are sensitized only by their specific hemolysin and this accounts for the highly specific nature of serum hemolysis. Sheep erythrocytes for example, are sensitized only by antisheep hemolysin and human erythrocytes only by antihuman hemolysin. But once sensitized the erythrocytes are hemolyzed by the one and same non-specific complement.

Hemolysins may be sometimes found normally or naturally in blood serum and are called *natural hemolysins;* they command considerable interest not only in relation to complement-fixation but likewise to blood transfusion. As examples may be mentioned natural antihuman hemolysin sometimes found in the serum of one human being for the corpuscles of another and called *iso-hemolysin;*

also natural antisheep, natural antiguinea-pig and other natural hemolysins in the sera of human beings and the lower animals. How these hemolysins are produced or why they should be present is not known. The amounts to be found in any serum however, are generally small; they are usually acquired during the first year after birth. It is possible that they are produced by the antigenic activity of certain non-specific lipoid-protein substances occurring in broken down human corpuscles and even in food substances similar to those first described by Forssmann in the tissues of the guinea-pig capable of producing heterophil antisheep hemolysin in rabbits, called heterophil antigens, and shortly to be discussed with more detail; in other words I believe that the natural hemolysins are heterophil in nature although nothing definite is known of the antigens engendering their production.

Hemolysins may be produced by injecting an animal with the erythrocytes of an unrelated species. Since these are the result of immunization they are called *immune hemolysins* and the best known are the antisheep, antihuman and antiox hemolysins produced by injecting rabbits with sheep, human or cattle erythrocytes respectively.

Small amounts of immune hemolysins however, may be produced by injecting rabbits with emulsions of various organs of the guinea-pig and some other of the lower animals. These were discovered by Forssmann[1] and have been called *heterophil hemolysins*. The phenomenon is a very curious one and not at all clearly understood at present, although bearing in some respects upon the mechanism of serum hemolysis and complement-fixation in general. In other words some tissues apparently contain a common antigen capable of engendering small amount of antisheep and possibly other hemolysins. This substance is also present in sheep erythrocytes and antisheep hemolysin is best produced by immunization with sheep erythrocytes particularly because the protein of the stroma is far more antigenic than the non-specific heterophil substance.

Complement is found in the blood of all animals but the amount varies greatly among different species and to a lesser extent among animals of the same species. It cannot be produced or increased by immunization and there is no such thing as an "immune complement" corresponding to immune hemolysin. Ehrlich and Morgenroth have insisted that a serum may contain several complements whereas Bordet states that there is but a single complement; as mentioned in the preceding chapter the phenomenon of complement-fixation was discovered by Bordet and Gengou while investigating this subject. The question has not yet been definitely settled but insofar as complement-fixation is concerned is largely of academic interest; Bordet's opinion however, is the one most generally accepted.

Experience has shown that the fresh serum of the guinea-pig is best suited for hemolysis, bacteriolysis and all other types of cytolysis and complement-fixation reactions; human serum complement ranks second in efficiency for these purposes.

When for example, washed sheep erythrocytes, antisheep hemolysin and guinea-pig serum complement are brought together in suitable proportions in a test-tube, the first thing to happen is a union of the antibody or hemolysin with the corpuscles. The sensitized cells then fix or absorb the complement, which gradually produces hemolysis. The union of hemolysin and erythrocytes does not alter the morphology or appearance of the cells and does not produce hemolysis. Complement alone does not unite directly with the corpuscles or cause their hemolysis. Only when the cells have been prepared or sensitized by the antibody or hemolysin, is complement, the lytic agent, absorbed and hemolytic. The phenomenon is exactly the same with the erythrocytes of other animals like those of man or ox except that the corresponding specific hemolysins must be used. A single serum may however, contain both hemolysin and complement. For example, fresh unheated human serum usually causes the hemolysis of sheep erythrocytes because it contains complement and a small amount of natural antisheep hemolysin. Likewise after rabbits have been injected with sheep corpuscles, their fresh unheated sera will hemolyze sheep erythrocytes in the test-tube because of the presence of the complement normally or naturally in rabbit blood plus the hemolysin engendered by the immunization; however, the amount or degree of hemolysis is slight because the serum does not contain enough complement to hemolyze the large number of corpuscles prepared or sensitized by the hemolysin.

SPECIFICITY OF HEMOLYSINS.

Hemolysins are highly specific for their corresponding or homologous antigen of erythrocytes. For example, antisheep hemolysin will sensitize or prepare only sheep erythrocytes for the lytic action of complement.

Natural hemolysins are more specific than immune hemolysins. Thus the natural or iso-hemolysin present in the serum of a human being for Group I human erythrocytes (Jansky) corresponding to Group IV (Moss), will not sensitize the corpuscles of the other three groups. But if a rabbit is immunized with Group I erythrocytes, the resulting immune hemolysin will sensitize equally or almost as well the corpuscles of all groups, as shown by Miss Trist and myself.[2] Likewise a rabbit immunized with the erythrocytes of one sheep will sensitize the corpuscles of all sheep although one occasionally encounters the corpuscles of a sheep which are not sensitized as com-

pletely as occurs with the erythrocytes of the majority of animals. Why this should occur I am unable to state. Naturally one first suspects that failure of hemolysis is due to deficient complement but I have satisfied myself on numerous occasions that this is not the case. Nor is it due to the fact that the corpuscles fail to absorb hemolysin, as I shall refer in a later chapter to experiments upon this peculiar and fortunately uncommon phenomenon.

NATURE OF ERYTHROCYTIC AND FORSSMANN HEMOLYSINOGENS.

Haptenes.—We may now properly inquire into what constitutes the active antigenic constituents of erythrocytes and other tissues capable of engendering the production of hemolysins; these substances may be designated as *hemolysinogens* since this word expresses their particular antigenic activity.

Insofar as erythrocytes are concerned it would appear to be the consensus of opinion that the protein of the stroma is most active; this is indicated by the results of investigations by Thiele and Embleton,[3] Balls and Korns,[4] Vedder,[5] Rule and myself[6] and numerous others. Whether or not the hemoglobin is capable of engendering the production of hemolysins is still a debatable question despite a large amount of work devoted to its elucidation. I believe it may be stated that crystallized hemoglobin is but feebly or doubtfully antigenic at best insofar as the production of hemolysin is concerned, although as shown by Heidelberger and Landsteiner,[7] it is capable of producing a species specific precipitin which is further evidence indicating that the antigenic substances producing lysins on the one hand and agglutinins and precipitins on the other may not be identical. Bang and Forssmann[8] and others have long maintained that the lipoids of erythrocytes were also capable of engendering the production of hemolysins but the consensus of opinion at present is against this view insofar as protein-free lipoids are concerned. As shown by Landsteiner rabbits injected with alcoholic extracts of corpuscles may sometimes produce small amounts of hemolysin but these results are ascribed to the presence of proteins in such extracts. The proteins of stroma therefore are to be regarded as the chief hemolysinogens but, as I shall discuss at greater length in Chapter IX, the corpuscles of different animals vary greatly in their antigenic activity, that is, in their property of engendering the production of hemolysin. For example, rabbits injected with sheep or ox corpuscles produce large amounts of hemolysin but very much less when injected with human, chicken or guinea-pig corpuscles.

The production of antisheep hemolysin by injecting rabbits with such heterophil or Forssmann antigens as extracts of the liver and

kidney of the guinea-pig, is largely due to a protein-lipoid complex; at least extracts are most antigenic while the purified lipoids of these tissues are practically without hemolysinogenic activity. But almost nothing more is known regarding the nature of these Forssmann hemolysinogens contained in the organs and tissues of the guinea-pig, horse, rat and some other of the lower animals while absent from those of the rabbit and man; that is to say we do not know whether or not they are contained in any particular cells of the various organs known to be antigenic as we have reason to believe that the stroma and not the hemoglobin of erythrocytes are mainly antigenic. It is to be emphasized again, however, that the hemolysinogenic activity of this non-specific or Forssmann substance is very much less than that of the stroma of the corpuscles of most animals. I may also mention that Hyde[9] reports the presence of heterophil antigen in chicken corpuscles since the injection of these into rabbits resulted in the production of antisheep and antigoat as well as antichicken hemolysins. I have been able to confirm this work. The sera of normal rabbits containing traces of natural antisheep hemolysin showed the presence of hemolysin for sheep corpuscles in titers as high as 1 to 1000 after six intravenous injections of 5 cc. of 10 per cent suspensions of washed chicken corpuscles. None of these animals showed the presence of natural antichicken hemolysin and after the injections the titer of immune hemolysin for chicken corpuscles was only 1 to 15. The hemolysinogens therefore, may not be strictly specific but the specificity dependent upon the kind of corpuscles and kind of animal used in their production.

But while lipoids and lipoid-protein fractions of erythrocytes and other cells are so poorly antigenic when injected into the lower animals that the resulting hemolysin production is almost negligible, yet these fractions may participate in precipitation and complement-fixation reactions in the test-tube in a very important manner. In other words the proteins of a substance are most antigenic *in vivo* but the lipoids or lipoid-protein complexes are sometimes of equal or greater importance in antibody reactions *in vitro*. *There may be a very important difference therefore, between the nature of antigens capable of stimulating the production of antibodies and antigens capable of giving reactions in the test-tube.* It is practically certain that when we inject washed sheep erythrocytes into a rabbit, the protein of the stroma engenders the production of the antisheep hemolysin, but at the same time it would appear that an additional protein-lipoid substance of the erythrocytes engenders the production of a second antibody capable of producing a precipitate in the test-tube when mixed with an alcoholic extract of sheep erythrocytes, as shown by Landsteiner and van der Scheer.[10] Apparently the lipoids alone of the corpuscles cannot engender the production of either

hemolysin or this precipitating antibody but lipoids may act as "antigens" in the test-tube in precipitation and complement-fixation reactions. Thus, as previously stated, Landsteiner and van der Scheer have observed precipitation reactions when alcoholic extracts of sheep erythrocytes were mixed with specific antisheep serum and Miss Yagle and I have been able to confirm their observations. Taniguchi[11] and others have also observed that the substance in extracts of guinea-pig liver or kidney capable of absorbing heterophil or Forssmann antisheep hemolysin resides in the lipoids of these tissues, especially in those soluble in alcohol and ether; furthermore, the antibody is capable of precipitating them and fixing or absorbing complement, the addition of cholesterol increasing both capacities.

It would appear therefore, that in serum hemolysis the antibody or hemolysin is best produced by immunization with the proteins of the antigen but in the test-tube an interaction may occur between an antibody and the lipoid or lipoid-protein portions of the antigen. In other words the lipoids of erythrocytes are not antigenic *in vivo* but may be "antigenic" *in vitro* in the sense of being precipitated by some kind of antibody in immune serum. Miss Yagle and I have found that the precipitation occurring when an alcoholic extract of sheep corpuscles is mixed with antisheep serum according to the method of Landsteiner and van der Scheer does not remove more than a trace of the hemolysin, showing that the latter has no relation to the precipitin of the lipoid-protein substances of erythrocytes. I mention these facts here, not because they have an important bearing upon the mechanism of serum hemolysis, but to show the difference between an antigen *in vivo* and *in vitro*, which in this case has an important analogy in the use of alcoholic extracts of such tissues as beef heart muscle as "antigen" in the complement-fixation and precipitation tests for syphilis. I will discuss these relationships more fully in the succeeding chapter but it may be stated at this time that we use the term "antigen" too loosely; it should be reserved only for designating substances capable of engendering the production of antibody.

But how shall we designate "antigens" *in vitro*, that is, substances like the lipoids of erythrocytes and other tissues capable of giving an antibody reaction in the test-tube although apparently unable to produce antibody in the living animal? Landsteiner and Simms[12] have proposed the term "haptenes" for their designation but I suppose the word "antigen" has been so long and widely employed for designating the substance capable of uniting with antibody in the test-tube that it will continue to be used in this way even though it is clearly realized that it may be a misnomer and especially so in the case of such substances as alcoholic extracts of beef heart capable of giving reactions in the test-tube with a syphilis antibody but yet totally unable to produce this antibody when injected into animals.

PROPERTIES OF SERUM HEMOLYSINS.

The immune hemolysins are highly resistant to destruction by heat, age, putrefaction, chemical agents and desiccation. But some of the natural hemolysins are quite susceptible to these agencies, especially those occurring in human serum for human, guinea-pig, rabbit and hog corpuscles. Natural antisheep hemolysin however, is much and antiox hemolysin slightly more resistant to heat and other destructive agencies.

None of the immune hemolysins undergo more than a very slight deterioration when heated at 55° C. for thirty minutes but at temperatures above 65° C. they begin to disappear and may be totally destroyed by boiling. Natural antisheep hemolysin is almost as resistant, but the natural hemolysins in human serum for human and guinea-pig corpuscles are so susceptible and especially the latter, that they may be inactivated at 55° C.

Immune hemolysins may be kept for years at ordinary refrigerator temperature with but very little deterioration. The serum may become heavily contaminated with bacteria and yet the antibody will resist destruction for long periods. The addition of such preservatives as phenol and glycerol does not cause destruction and the serum may be dried and ground to a powder or dried in filter paper with remarkably little destruction or inactivation of the hemolysin. Shaking does not harm them as it may complement; they are also resistant to roentgen-rays but regarded as susceptible to ultra-violet rays. They are readily passed through Berkefeld filters but are not usually dialyzable.

They are fairly resistant to acids and alkalies. Miss Yagle and I have observed that 1 cc. of 1 to 10,000 rabbit antisheep hemolysin is destroyed by 1 cc. of 1 to 15 dilution of normal sodium hydroxide in an exposure of one hour at 38° C. and by 1 cc. of 1 to 400 of a normal solution of hydrochloric acid; these amounts correspond approximately to 0.0026 gm. of sodium hydroxide and 0.0001 cc. of hydrochloric acid. Complement is far more susceptible to destruction by these substances, as I shall discuss in Chapter VIII in relation to the influence of acids and alkalies in the saline solution and upon serum hemolysis and complement-fixation. Strong sodium chloride solutions do not prevent the union of corpuscles and hemolysin although they may interfere with hemolysis by action upon complement.

NATURE OF SERUM HEMOLYSINS.

Serum hemolysins may be defined as antibodies capable of uniting with their homologous erythrocytes and rendering them sensitive or susceptible to the lytic effects of complement. But while this definition

states what they do it does not indicate what they are. In truth their exact nature is unknown although from the chemical standpoint it is practically certain that they belong to the globulins.

By fractionation methods it has been well established, for example, that they are carried down in the globulin portion of immune serum, as is true of all antibodies in general; but this does not necessarily identify them as globulins. When sheep corpuscles are injected into a rabbit an antisheep hemolysin is produced; when this immune rabbit serum is injected into a sheep an antiamboceptor or antihemolysin serum is produced capable of inhibiting the hemolytic activity of the rabbit serum for sheep corpuscles. This has been accepted as evidence of the protein nature of hemolysin because it is capable of engendering the production of an antibody against itself. But if the sheep is injected with normal rabbit serum it is found that the sheep serum is likewise capable of inhibiting the hemolytic properties of rabbit antisheep hemolysin. In other words it would appear that a sheep injected with either normal or immune rabbit serum produces an antirabbit serum which, when mixed in a test-tube with rabbit antisheep serum, produces a precipitation of proteins, including the globulins, and thereby removes or in some way inhibits the activity of the hemolysin. This is presumptive evidence of the protein nature of hemolysin but until we are able to isolate it in a pure state it is impossible to determine its chemical nature exactly or to state whether a true antihemolysin can be produced by injecting it into an animal.

But some progress has been made in this direction. The early attempts of von Lieberman and Fennyvessy[13] and Rondoni[14] using diluted hydrochloric acid and sodium hydroxide respectively, for dissociating hemolysin from sensitized corpuscles, failed to isolate the antibody because of the effects of these substances upon the erythrocytes. Kosaki[15] however, has been more successful with isotonic and hypertonic watery solutions of saccharose, glucose and lactose but has not reported upon the results of chemical studies with the isolated hemolysin. Uchida,[16] in my laboratory, has repeated Kosaki's experiments with some success, although the amount of isolated antisheep hemolysin was very small. But Uchida obtained sufficient amounts for various protein tests similar to those employed by Huntoon and his associates in a study of isolated pneumococcus antibody and found that the solutions were not digested by trypsin, did not sensitize guinea-pigs or yield the usual protein color and precipitating reactions. Ottenberg[17] has tried to isolate hemolysin by precipitating the immune serum with the chlorides or sulphates of copper, nickel, zinc, mercury and lead followed by dissolving out the antibody from the precipitates, but on account of the large amount of protein present it has not been found possible to really secure the antibody in a pure state. Locke

and Hirsch[18] employing a process of electro-dialysis unassisted by iso-electric fractionation succeeded in isolating hemolysin of such high purity that each hemolytic unit contained but 0.000002 gm. of protein and some lipin. They observed that when the lipins of sensitized corpuscles are partially removed by extraction with ether, the stroma-hemolysin combination is loosened and rendered easily dissociable along with a shifting of the iso-electric point of the stroma from approximately a pH 5 to pH 7. By means of this method hemolysin was isolated in such purity that each unit contained but 0.000125 to 0.00018 mg. of protein. They have also proposed qualitative methods which under restricted conditions permit the detection of protein in concentrations as low as 0.006 to 0.035 mg. per cubic centimeter.

I believe that the general results of these studies have been to show that the hemolysins, like antibodies in general, are proteins belonging to either the pseudo-globulin or euglobulin fraction of serum. Huntoon has reached this opinion regarding the nature of isolated pneumococcus antibody, since he has been able to concentrate his solutions for chemical study and guinea-pig sensitization tests. Certainly the purified lipoids of immune serum do not carry the specific hemolysin even though they, as well as the lipoids of tissues and bacteria, may sometimes hemolyze corpuscles direct and in a non-specific manner. Several investigators have sought to identify the serum hemolysins with the lipases or lipoids of the serum but without particular success. I have extracted 1 cc. amounts of rabbit antisheep hemolysin, possessing a titer of 1 to 10,000, with 15 cc. amounts of alcohol, ether and acetone; these extracts have been evaporated and the residues, dissolved or suspended in 1 cc. of saline solution, tested for hemolytic activity; the alcohol and acetone extracts showed a titer of 1 to 32 and the ether extract a titer of 1 to 3000 when tested for hemolysin in the presence of complement. None of the extracts alone were hemolytic for washed sheep cells in dilutions of 1 to 2. The results, however, were probably due to the presence of proteins in the extracts carrying hemolysin since further purification of the lipoids resulted in reducing hemolytic activity. The carbohydrates of serum may be likewise excluded so that by exclusion alone we may conclude from the chemical standpoint that the hemolysins are protein substances.

But what are hemolysins? Are they enzymes? What essential change do they produce in erythrocytes and how do they act with complement to produce hemolysis? These are questions to be considered shortly in a discussion of the mechanism of hemolysis but it is to be admitted at the outset that their exact nature is unknown. As previously stated they do not appear to have a relation to the precipitin in immune serum for the lipoid-protein substance contained in alcoholic extracts of corpuscles since Miss Yagle and I have

observed that when this precipitin is removed from antisheep serum none or but a trace of the hemolysin has disappeared in the reaction and precipitate. Hill and Parker[19] have recently advanced the hypothesis that hemolysin is a ferment or contains a ferment which is activated in sensitized corpuscles by complement. But I believe it more likely that the process is just the reverse and that complement is a ferment or enzyme activated by hemolysin. Certainly the immune hemolysins do not possess as many of the properties of enzymes and if serum hemolysis is an enzymic reaction the hemolysin may be regarded as some kind of activator for the lytic complement.

PROPERTIES OF COMPLEMENT.

In sharp contrast with the hemolysins, complement is very susceptible to various physical agencies. When a serum contains complement it is said to be *active* and this must, under ordinary conditions, be a fresh serum. It deteriorates rapidly after bleeding unless the serum is frozen or otherwise specially preserved. At ordinary room temperature deterioration is rapid within twenty-four hours, and generally complete within a few days. When dried at ordinary room temperature its activity is lost either at once or within a few days; however, when dried at very low temperatures in a vacuum, the activity may be quite well preserved, as I shall discuss in Chapter XI, when taking into consideration various methods for the preservation of guinea-pig serum complement.

Probably the most prominent characteristic of complement is its extreme sensitiveness to heat since exposure to 55° C. for ten or fifteen minutes results in complete *inactivation*. This is commonly believed to be due to destruction of the cytolytic portion or that part of the molecule concerned in hemolysis; what is left is designated as *complementoid* and believed capable of uniting with sensitized erythrocytes and thereby preventing or reducing hemolysis by blocking the union of active complement with the sensitized cells.

Complement may be also inactivated by violent shaking but since Noguchi and Bronfenbrenner[20] have shown that shaking of guinea-pig serum for one hour at 37° C. was almost without influence, it may be concluded that the several brief shakings employed in conducting complement-fixation tests have no appreciable influence upon the reactions.

But inactivation by heat may not completely destroy complement since, according to Gramenitz,[21] when 1 to 10 guinea-pig complement is heated at 56° or 57° C. for a few minutes it may regain activity after cooling; this is ascribed to spontaneous regeneration and regarded as additional evidence of the ferment or enzyme nature of complement. Others however, believe that this indicates that

complement is a hydrophilic colloid, if it is assumed that heating causes a reduction in surface tension and a consequent aggregation of the particles which, if not carried too far, may be reversible and followed by dispersion with gradual restoration of the colloid to an active state. Further evidence of the colloidal nature of complement is indicated by its filterability. Plain guinea-pig serum complement does not ordinarily pass through a Berkefeld filter until enough serum has been forced through to saturate the absorptive capacity of the filter which indicates the large size of the molecule. According to Muir and Browning[22] the addition of 5 per cent sodium chloride increases its filtration and on dilution its activity is restored, but the nature of these effects of salt are not understood.

The activity of complement requires the presence of sodium chloride and saturation of serum with this salt aids in its preservation. But, on the other hand, it readily unites with the ions of magnesium, calcium, barium and such substances with inactivation, as shown by Hektoen;[23] Manwaring[24] found that these ions could be separated again by simple chemical precipitation. Acids stronger than CO_2 and of the higher saturated and unsaturated fatty acid series, readily destroy complement and especially, as shown by Brooks,[25] when the pH concentration passes the iso-electric point. Alkalies are likewise destructive or anticomplementary and for these reasons the saline solution and glassware employed in complement-fixation tests must be prepared with due care and all extracts employed as antigens, like alcoholic extracts of beef heart or other tissue used in the Wassermann test, carefully tested or titrated for the presence and activity of these anticomplementary or complement-inactivating substances. It is also possible as shown by Sherwood,[26] that various substances present in excessive amounts in human blood during pathological conditions, such as carbon dioxide, lactic acid, uric acid, acetone, etc., may interfere with the activity of complement, but I shall discuss these in a later chapter when considering the influence of blood chemistry upon the specificity of the Wassermann and other complement-fixation tests.

NATURE OF COMPLEMENT.

This now brings us to a consideration of the nature of complement and it may be stated at once that it is still one of the many debated riddles of immunology. *Complement may be defined as a substance present in both normal and immune sera which produces lysis of sensitized antigen.* In the case of serum hemolysis it is the substance in guinea-pig serum which causes the lysis of sheep corpuscles when they have been first prepared or sensitized by the specific antisheep hemolysin. However, while this defines what complement does it states nothing concerning its nature.

It is now generally accepted that complement is a protein or at least is carried along with the proteins of serum as shown by the results of a large amount of investigation devoted to the subject of "complement-splitting." This means that by dialyzing the serum against distilled water for twenty-four hours to remove the salts, that one part of complement is contained in the precipitated globulins and called the "mid-piece" while another portion is contained in the water-soluble albumins and designated the "end-piece." It is commonly stated that the "mid-piece" or globulin fraction will unite with sensitized cells but is unable to produce lysis until the albumin fraction or "end-piece" is united; Whitehead, Gordon and Wormall[27] have recently stated that even a fourth or heat stable component may exist, not identical with the third component. Bronfenbrenner and Noguchi[28] however, contend that all of the complement is contained in the albumin fraction but the subject hardly requires further discussion in relation to serum hemolysis and complement-fixation beyond my purpose to show that complement is identified with the proteins of serum and probably largely with the globulin fractions, although Brooks[29] has recently stated that this could not be the case because complement exposed to ultraviolet light is not thereby sensitized to heat as is the case of proteins. He advances the hypothesis that there is present in serum a hemolytic substance which is formed from a precursor resembling lecithin, constantly formed and simultaneously broken down into inactive products, but the activity of the lytic agent is dependent on the state of the serum colloids. More recently Locke, Main and Hirsch[30] have advanced a somewhat similar hypothesis as follows: "The nature of the hemolysin-erythrocyte equilibrium indicates that there is in amboceptor serum a substance, probably derived from the injected antigen, which is capable of binding hemolysin and restraining its adsorption upon added cells. The degree of the characteristic properties of an immune serum, such as specificity, avidity, and thermostability is shown to be dependent upon the amount and character of this binding substance dispersed in the serum. The substance is absorbed by erythrocytes together with hemolysin. When hemolysin is recovered from adsorption, its protein content is roughly equal to the sum of a constantly associated quantity of protein plus the calculated protein content contributed by the binding substance. The properties of the hemolysin may be partly changed by changing the proportion and character of its binding substance. It is postulated that antibodies are composed of clusters, of relatively large dimension, in which an elementary, naturally occurring, protein substance is adsorbed upon nuclei of a binding substance derived from the antigen, and that they owe their individual properties to the proportion and character of this binding substance."

Hecht[31] also regards the complement of serum as only the property of a mixture of colloids and electrolytes in water and dependent for activity upon such factors as the surface tension, viscosity, conductivity and degree of dispersion; in his opinion complement function is the combined action of several factors of which globulin-colloid (mid-piece), albumin plus lipoid colloid (end-piece) and electrolytes, constitute the best combination. A change in the physico-chemical state of any one of these factors, *e. g.*, lowering the surface tension by freezing, or disturbing the conductivity by alcohol, distilled water, etc., inactivates the complement as a whole. Schmidt[32] also subscribes to the view that inactivation by shaking is the result of an alteration of the colloidal state of the serum rather than the result of a chemical change. Kiss[33] believes that complement is composed of hydrophilous colloids which, however, cannot be divided without losing their characteristic properties. He states that all forms of complement-fixation follow the rules of physical adsorption, and hence must be regarded as a physico-chemical process. According to this view therefore, complement is adsorbed by corpuscles and particularly when their absorbing capacity is increased by specific hemolysin but how the specificity of the reaction is to be explained on this simple physical basis has not been shown. However, it is well established that complement is adsorbed by various precipitates, silicic acid, gum mastic and other colloidal aggregates, especially at low temperatures, which tends to place the phenomenon of hemolysis and complement-fixation upon a colloidal physico-chemical basis rather than upon an enzymic basis.

As previously stated complement is a normal constituent of the blood and as such probably serves a vital function in chemical changes including both metabolic and catabolic changes accompanying growth. Antibodies are ordinarily produced by the body cells to prepare new and invading substances for destruction by this normal blood substance which probably plays a continuous and important role in the final metabolism of foods after their absorption into the lymph and blood. I believe that complement is a proteolytic enzyme despite the fact that it seems to work according to the law of definite proportions, unlike the enzymes. In other words it seems to be used up during lysis whereas the enzymes as we understand them are still recoverable after proteolysis. But as shown by Liefmann and Cohn[34] the disappearance of complement may be only apparent rather than real and due to masking by secondary processes, just as Northrop[35] has recently shown that pepsin combines with the products of hydrolysis and maintains an equilibrium with these according to the laws of mass action, only the uncombined residual pepsin continuing to be active. In such proteolysis the pepsin is used up, just as complement is used up in hemolysis. Certainly Buchner, Bordet, Metchnikoff, Ehrlich

and Morgenroth and numerous others have regarded complement as enzymic in nature, the leukocytes being commonly regarded as one source of its production, and a careful review of the evidence has led Liefmann[36] and others to the conclusion that the action of complement upon sensitized erythrocytes is like that of a ferment upon substrate. It is true, however, that in hemolysis the stroma and hemoglobin appear to remain intact and Browning,[37] Wollman and Graves,[38] McNeil and Kahn,[39] and others have failed to find such products of proteolysis as an increase of peptones and amino-acid nitrogen; but yet by delicate methods and especially by the use of ninhydrin for the detection of the alpha-amino-acids, some evidences of digestion may be obtained and I believe sufficient to account for enough digestion of stroma to liberate unchanged hemoglobin. Certainly the proteins of the stroma appear to be the chief and possibly the only antigen of erythrocytes engendering the production of hemolysin and I believe they undergo the main change in serum hemolysis.

The evidence, therefore, for regarding complement as an enzyme may be briefly summarized as follows:

1. When corpuscles are sensitized with one unit of hemolysin, the activity of complement depends on its concentration and not on the total amount present. When, however, the corpuscles are sensitized with an excess of hemolysin, this holds after a certain degree of dilution as shown by Scheller,[40] Thiele and Embleton[41] and others; the same relations have been observed in the action of trypsin on gelatin. In other words complement conforms with the law of mass action of enzymes.
2. The curves of complement activity are similar to those of enzyme action.
3. Complement, like the enzymes, does not dialyze through collodion or other suitable membranes or does so but very slowly.
4. Alcohol and acetone precipitate complement as they precipitate most of the enzyme preparations from their solutions.
5. Sodium chloride is necessary for complement activity as it is for amylase and some other enzymes. Removal of this salt inactivates complement as it does some other enzymes.
6. The activity of complement is greatly influenced by the hydrogen-ion concentration of the medium which likewise influences the activity of enzymes in general.
7. Inorganic salts greatly influence complement activity usually by retardation just as they may accelerate or retard the activity of enzymes. This bears an important relation to the inorganic salts contained in water used in the preparation of saline solution, with or without buffers, as I shall discuss in Chapter VIII.
8. Complement gradually deteriorates even at comparatively low temperatures as do the enzymes in general.
9. Complement is rapidly inactivated at 55° C. as are the enzymes. This is apparently due to chemical changes within the molecule, other changes like coagulation being secondary processes.
10. The activity of complement is greatest around 38° to 40° C. as is true of the enzymes in general.

It is true that complement is lacking in the specificity of action somewhat characteristic of the enzymes but it may be, as maintained by Ehrlich, that a serum contains numerous complements more specific for different lytic processes than may be assumed on the basis of Bordet's view that serum contains but one complement.

Finally the enzymic nature of complement in no way underestimates the importance of colloidal factors. As a matter of fact if complement is an enzyme it exists as a colloid because practically all enzymes are produced by living matter and exist as such. If complement has never been isolated in a chemically pure state the same may be said of all enzymes. Certainly it would appear to be of a protein nature and the same is true of all enzymes since it is known that they contain nitrogenous bodies and especially amino-acids linked in a peptide combination.

While Noguchi,[42] von Liebermann[43] and others have shown that soluble oleates mixed with serum possess some of the properties of complement, which led to the opinion on the part of some that complement was lipoidal and identified with the soaps of serum, yet it is now quite generally accepted that they are not identical with true complement. Certainly von Liebermann's artificial complement composed of heated normal rabbit serum and methyl alcohol solutions of sodium oleate and calcium chloride, cannot be used as a substitute for serum complement in the Wassermann test, as shown by Schmidt[44] and likewise observed in my own experiments with it; Gramenitz has also observed that it does not regain activity upon cooling after heating for a few minutes at 56° C. which he regards as evidence indicating the enzymic nature of true serum complement. As previously mentioned Hill and Parker regard complement as a catalyst, allowing the release of hemoglobin by the action of a ferment in the hemolysin. But in my opinion it is more plausible to regard complement as a ferment activated by hemolysin.

AUTHOR'S THEORY OF THE MECHANISM OF SERUM HEMOLYSIS.

Obviously with so many conflicting opinions regarding the nature of specific hemolysins and complement it is difficult to define the exact mechanism of serum hemolysis. That the hemolysin must first unite with the corpuscles and sensitize them to the effects of complement is well proven, as likewise the fact that the hemolysin alone and complement alone are without apparent injury to the cells. The union of hemolysin with corpuscles does not appear to injure them at all. With just what portion of the cell this union occurs is not definitely known; the Mudds[45] have recently stated that the increased polarity of the sensitized erythrocyte surface is due to a

combination of the red cell surface lipoids with the hemolysin; but while this observation may bear a relation to the precipitation of erythrocytic heterophil antigen in the test-tube it apparently does not have a relation to the mechanism of serum hemolysis. It is certain that the washed stroma alone are capable of absorbing the specific hemolysin and this is of more significance in relation to the mechanism of serum hemolysis.

As shown by Rockwood[46] the rims of the erythrocytes in serum hemolysis simply fade away without any swelling or other change in the cell volume. I have observed the same change without any evidence of intracellular precipitation insofar as these were detectable by dark-field microscopy. Agglutination of the cells appears to give them considerable protection against hemolysis. It is stated that the stroma shows no change but with this view I cannot agree; at least in the complete hemolysis of sheep corpuscles it would appear impossible to recover more than a fraction of the stroma by centrifuging and precipitation methods. The nuclei of hemolyzed chicken corpuscles, however, are much more readily recovered and do not appear to be affected.

In my opinion serum hemolysis is an intracorpuscular enzymic reaction in which the hemolysin renders the corpuscles susceptible to an enzyme in the complement and resulting in the digestion of stroma sufficient for the release of hemoglobin. Otherwise, the process may be regarded as an obscure physical colloidal phenomenon along the lines suggested by Brooks in which the complement of the blood is regarded as being constantly formed from a precursor (which may resemble lecithin) and simultaneously broken down to maintain a fairly uniform equilibrium, the hemolysin being regarded as a substance capable of altering the colloidal state of the serum proteins in such a manner as to activate the lytic complement. That physical agencies and particularly the pH concentration of the menstruum may influence the mechanism is well proven but this is not inconsistent with the enzymic nature of serum hemolysis and until colloidal chemistry has more definite information to offer consistent with the specificity of the process I would rather regard serum hemolysis as an intracorpuscular enzymic process.

But how does the hemolysin act? Is complement a pro-enzyme requiring activation by hemolysin? The nature of hemolysin and the mechanism of its action are even more difficult of elucidation than the nature and behavior of complement. In keeping with the hypothesis of the enzymic nature of serum hemolysis I believe that erythrocytes naturally resist destruction by complement enzyme through the presence in them of a specific anticomplement or anti-enzyme similar to the antienzymes of all living cells in general.

When corpuscles are injected into an alien animal there is produced a substance called the antibody, amboceptor or hemolysin

which, coming in contact with the corpuscles neutralizes the inhibitory effects of their antienzyme and thereby renders the cells sensitive or susceptible to the digestive and lytic effects of the complement enzyme. I believe the same mechanism is true of bacteriolysis and other types of specific serum cytolysis.

In titrating hemolysin by using varying amounts of immune serum with constant amounts of complement and corpuscles, it is sometimes observed that hemolysis does not occur at all or is less complete in tubes carrying the largest amounts of hemolysin whereas hemolysis is complete in those tubes carrying the higher dilutions of hemolysin. This paradoxical zone or Neisser-Wechsberg phenomenon has been variously explained and especially on the assumption that the excess of hemolytic amboceptors unite direct with complement and thereby deviate it from union with sensitized corpuscles, which results in a failure of hemolysis.

But this explanation is inadequate. In the first place the phenomenon is always more likely to occur with old than with fresh serum and under these circumstances may be due to an anticomplementary condition of the serum. Secondly, it is more likely to occur with weak immune than with powerful immune sera and therefore does not bear a relation to the number of amboceptors present. Thirdly, furnishing an excess of complement to unite with the free amboceptors as well as the sensitized corpuscles does not always produce hemolysis although the phenomenon is most likely to occur when but small amounts of complement are present. Fourthly, it has been quite definitely proven that complement does not unite direct with free amboceptors. The phenomenon is much more frequently seen in bacteriolytic, the Wassermann and other complement-fixation reactions and will be discussed more fully in the succeeding chapter but insofar as serum hemolysis is concerned, I believe that the inhibition of hemolysis in those tubes carrying the largest amounts of immune serum is due to an interference by the salts or the proteins of the serum with those physico-chemical colloidal conditions required for precipitation and the full activity of complement.

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CHAPTER III.

PHENOMENON OF COMPLEMENT-FIXATION.

It is difficult to briefly *define* complement-fixation. In general terms *it is the fixation, absorption or inactivation of complement by an interaction between antigen and antibody.*

According to prevalent theories the antigen and antibody are supposed to unit in the test-tube and this complex, which may be accompanied by precipitation, is regarded as physically "fixing" or absorbing the complement. But it is not proven that the process is so simple. Rather, as discussed in Chapter VI, it may be that the interaction between antigen and antibody results in a disturbance of the chemistry of the mixture producing an inactivation of the complement. While complement-fixation may occur in the living animal, in practice the term is usually employed for reactions *in vitro* and is so employed in this treatise.

SPECIFIC AND NON-SPECIFIC COMPLEMENT-FIXATION REACTIONS.

Two kinds of complement-fixation reactions are recognized, namely, (1) the *specific complement-fixation reaction* in which complement is fixed or inactivated by a complex of specific antigen and specific antibody and (2) the *non-specific complement-fixation reaction* in which complement is fixed or inactivated by a complex of antibody with a non-specific extract of some kind designated for convenience as "antigen."

As examples of specific complement-fixation reactions may be mentioned the tuberculosis, typhoid, glanders, gonococcus and echinococcus reactions for the diagnosis of their respective diseases, employing extracts or suspensions of the organisms for the fixation of complement with the respective specific antibodies; also such reactions as those employing antigens of soluble albuminous substances like blood stains, milk, meat extracts, etc., with their corresponding specific antibodies.

The Wassermann reaction however, is the best known and most striking example of a non-specific complement-fixation reaction because the extract employed as "antigen" is ordinarily prepared of beef heart or some other tissue entirely unrelated to the real antigen of syphilis, namely, *Spirochæta pallida*. The same is true of the Sachs-Georgi, Meinicke, Kahn and other precipitation reac-

tions in syphilis which likewise employ extracts of normal non-syphilitic tissues as "antigens." Furthermore the complement-fixation reactions sometimes observed with the sera of *normal* rabbits, dogs and mules with extracts of various tissues or bacteria employed as so-called "antigens," are additional examples of non-specific reactions.

Complement-fixation versus Complement-deviation: Terminology.— Complement-fixation refers to the fixation or inactivation of complement by a complex of antibody and antigen. "Complement-deviation" is a term coined by Neisser and Wechsberg to designate the apparent fixation of complement by antibody alone. According to their theory if a serum contains a great excess of antibody some of the complement may be fixed by the antibody alone and thereby become "deviated" or side-tracked from fixation by the antigen-antibody complex. But it has not been proven that antibody and complement are capable of direct union and the phenomenon of Neisser and Wechsberg is explainable on the basis of a zone reaction involving the colloidal chemistry of complement reactions. Thjøtta[1] has recently expressed the opinion that during infection or artificial immunization specific inhibiting antibodies may develop which interfere with the fixation of complement by the antigen-antibody complex and thereby exhibit the Neisser-Wechsberg phenomenon but Pandit[2] denies this possibility and believes that the reaction is the result of a dissociation of the antigen-antibody complex which occurs when the latter is in excess thereby leaving the complement free in the mixture. I shall have occasion to refer to this zone phenomenon again in Chapter VI because it is sometimes encountered in the syphilis and other complement-fixation reactions when a large amount of serum like 0.1 to 0.2 cc. yields a weaker reaction than smaller amounts, but at any rate the original meaning attached to the phrase "complement-deviation" does not hold at the present time and I believe that it may be properly used as a synonym for complement-fixation; this is indeed quite a common practice.

The complement-fixing antibody is probably best known under the term "amboceptor" introduced by Ehrlich because he conceived it as a substance possessing a double action namely, one for specific union with its antigen and the second for union with complement. A second well known term is "sensitizer" coined to meet Bordet's idea that the antibody sensitizes or prepares the antigen followed by the fixation of complement. I think it is a much better term than amboceptor but the latter is better known and more widely employed. Several years ago[3] I proposed the term "alexofixagin" because it more accurately defines the antibody as one capable of fixing alexin or complement. However, the antibody may be identical with precipitin, a subject to be discussed with more detail in

the succeeding chapter, although at present most immunologists apparently regard these as separate and distinct antibodies.

In a strict immunological sense "antigen" means a substance capable of engendering antibody production. But the word has also come into general use for designating the extract or substance placed in the test-tube with serum for union with the antibody and the fixation of complement. In relation to the specific reactions this usage is permissible but in the non-specific reactions it is not. For example, in the gonococcus complement-fixation test the antigen is a suspension or extract of the gonococcus which is also capable of producing the antibody if injected into human beings or the lower animals, but in the Wassermann test the so-called "antigen" is usually an alcoholic extract of beef heart or some other tissue which is not capable of producing the Wassermann antibody upon injection. However, as stated in the preceding chapter, the word "antigen" has come into very general use in complement-fixation work and doubtless will continue to be used more commonly than such substitute terms as "extract," "emulsion," "suspension," "haptenes," etc.

Complement-fixation versus Complement Destruction; Anticomplementary Activity.—Complement is very easily destroyed or inactivated as already discussed on page 33 and in complement-fixation tests the glassware may contain sufficient amounts of acid or alkali to destroy this reagent; extracts employed as antigens and particularly large amounts of alcoholic extracts of tissues used in the Wassermann test, may contain sufficient alcohol, fatty acids, etc., to have the same effect. The results are a partial or complete inhibition of hemolysis, depending upon the degree of destruction of complement, which, under some circumstances, may be regarded as a positive complement-fixation reaction; when this occurs in the Wassermann test it may prove a very grievous error. Anything destroying the activity of complement, therefore, may give an end result of interference of hemolysis looking exactly like a true positive complement-fixation reaction and great care is required in guarding against such errors.

Sera, spinal fluids, antigens or other fluids submitted to the complement-fixation test may contain thermolabile or thermostabile substances or colloidal changes which may not actually destroy complement but produce its fixation or inactivation alone, that is, in the absence of antibody; this phenomenon constitutes *anticomplementary activity* and is discussed more completely in Chapter VII because of the great practical importance of the subject. The reaction is called "anticomplementary" because it is easily shown that the substances in sera, spinal fluids and antigens responsible for it act upon complement and not upon the corpuscles or hemolysin. In other words these effects do not appear to be an actual destruc-

tion of complement as an acid or alkali may destroy it but merely an inactivation of that part which is concerned in serum hemolysis so that partial or complete inhibition of hemolysis results.

THE PHENOMENON OF SPECIFIC COMPLEMENT-FIXATION.

No better example of a specific complement-fixation reaction can be given than that originally devised by Bordet and Gengou,[4] which may be briefly summarized as follows:

1. The serum of a horse immunized with *B. pestis* (bubonic plague) and containing antibody was first heated at 55° C. for thirty minutes to remove its complement (called "inactivation of serum"); a small amount was then placed in a test-tube along with a small amount of a suspension of killed pest bacilli, called the antigen.
2. A second tube was set up in the same manner using inactivated normal horse serum containing no antibody.
3. A small amount of fresh guinea-pig serum (called alexin or complement) was added to each tube because it was believed that guinea-pig serum contained the best alexin or complement for the reaction and experience since then has confirmed this opinion.
4. An interval was allowed (called "primary incubation") to permit the union of the antibody with the antigen in the first tube which was followed by the fixation of complement. In the second tube the complement was not fixed because there was no antibody and the antigen alone in the amount used did not affect it.
5. But it was necessary to prove that complement was fixed in the first tube by the antibody in the immune serum and the antigen but not in the second tube by the normal serum and antigen. This could not be done by looking at the tubes as their appearance to the naked eye was the same after as before incubation. So it was necessary to conduct the second phase of the reaction to determine this point, which was done as follows:
6. Washed rabbit erythrocytes were mixed with antirabbit hemolysin or sensitizer (prepared by injecting rabbit blood into guinea-pigs) and a small amount of the mixture added to each tube.
7. This was followed by another period (the "secondary incubation") to allow unfixed complement to hemolyze the sensitized corpuscles.
8. But in the first tube hemolysis did not occur (*a positive reaction*) because there was no free complement since it had been "fixed" by the antipest antibody and pest antigen during the primary incubation. In the second tube however, hemolysis was complete (*a negative reaction*) because complement was present since it was not fixed during the primary incubation, there being no antipest antibody in the normal horse serum and the antigen of pest bacilli alone being unable to fix the complement.

This, therefore, serves as an example of a specific complement-fixation reaction because additional tests showed that only antipest antibody would fix complement with an antigen of pest bacilli. It also served as an example of the principles concerned because additional tests showed the following:

1. That the antipest serum alone did not fix complement.
2. That the normal serum alone did not fix complement.
3. That the antigen alone did not fix complement.

4. That hemolysis occurred in the tube carrying the rabbit corpuscles, antirabbit hemolysin and complement (called the "hemolytic system control tube") because the hemolytic system was complete and the hemolysin and complement active and present in sufficient amounts.

5. That hemolysis did not occur in a tube carrying corpuscles, hemolysin and saline solution (called the "corpuscle control") because complement was not present and the saline solution was isotonic.

Indeed the whole experiment was wonderfully correct in the technical details because all of the necessary controls were included and experience has since proven that these are always an absolutely essential part of every complement-fixation test because it must be *shown* and not assumed that the complement and hemolysin are active, present in sufficient amounts and capable of producing hemolysis; also that the serum being tested is not alone able to inactivate or destroy the complement and thereby interfere with hemolysis, which sometimes occurs in these tests (called the "anti-complementary action of serum"); also that the antigen alone is not able to inactivate or destroy the complement (called the "anti-complementary action of antigen") with a similar interference of hemolysis and either or both of which might be interpreted as specific positive reactions if the controls were not included to show the error.

Furthermore, it is always necessary to show that the antigen is sensitive and employed in sufficient amount by yielding a positive reaction when tested with a serum carrying its antibody (called the "positive control"); likewise that it does not give a falsely positive reaction when tested with a normal serum (called the "negative control"), that is, it must be free of anticomplementary effects in the dose employed.

THE PHENOMENON OF NON-SPECIFIC COMPLEMENT-FIXATION.

No better example of a non-specific reaction can be given than the original syphilis reaction developed by Wassermann and his colleagues in 1905 and independently and at the same time by Detre. Both used watery or saline extracts of syphilitic tissues for antigens and ascribed the positive reactions to a specific fixation of complement by syphilis antibody and *Spirochæta pallida* in the antigen extracts. But we now know that while such extracts *may* give a weak degree of specific fixation, yet unquestionably the reactions are due in larger part or totally to the presence of lipoids in the extracts (antigens) interacting with the syphilis antibody and resulting in the fixation of complement. It has been definitely

and surely proven that these lipoids are the active and essential factors in the antigen extract and that they may be obtained from healthy non-syphilitic tissues of man or the lower animals.

Therefore, the Wassermann-Detre reaction in syphilis is an example of non-specific complement-fixation because the extract employed as antigen is not specific since it is ordinarily an aqueous or alcoholic extract of tissue carrying lipoids entirely unrelated to syphilis. Practically however, the Wassermann reaction is highly specific because the peculiar antibody responsible for the reaction occurs with certainty only in syphilis and frambesia tropica (yaws.)

For this reason I naturally dislike mentioning the Wassermann reaction as an example of non-specific complement-fixation but from the biological sense this is true, just as the Kahn, Sachs-Georgi and the host of precipitation tests in syphilis are non-specific for the same reason.

Probably a purer example of non-specific complement-fixation is that seen when heated *normal* rabbit serum is submitted to the Wassermann test. The serum apparently contains no syphilis antibody since it is taken from a perfectly healthy and normal rabbit; neither is it anticomplementary. Yet in the presence of a Wassermann or bacterial antigen used in the proper dose, well-marked complement-fixation sometimes occurs.

The set up of these tests is exactly the same as described above for the specific complement-fixation test; the non-specific element in the Wassermann test enters because the so-called "antigen" is entirely unrelated to *Spirochæta pallida* and in the case of rabbit serum because the antibody-like substance is apparently unrelated to the "antigen" employed.

SCOPE OF THE COMPLEMENT-FIXATION TEST.

From the standpoint of serum diagnosis the complement-fixation test may be used in two ways as follows:

1. *If the Antigen is Known the Antibody May be Detected.*—For example, in the Wassermann test an extract is employed as "antigen" which is known to be capable of interacting with syphilis antibody, if this is present in the serum or spinal fluid being tested, followed by the fixation of complement. Under suitable conditions a positive reaction indicates the presence of the antibody and this in turn the presence of *Spirochæta pallida* in the tissues (providing the possibility of yaws is excluded and the test is technically correct). Similar examples may be multiplied at length. One may use an antigen of the gonococcus for the detection of gonococcus antibody; or an antigen of the typhoid bacillus for the detection of typhoid antibody, etc. Under acceptable technical conditions the positive reactions are highly specific, being based upon the laws of

antigen-antibody reactions, and are of considerable practical value in diagnosis.

2. *If the Antibody is Known the Antigen May be Detected.*—This is the reverse of the above. For example, one may immunize a rabbit with human blood and thereby produce antihuman serum containing a known antibody. This serum may be added to complement and an extract of blood of unknown source; under suitable technical conditions a positive reaction shows that the blood was of human origin.

In this manner the complement-fixation test may be employed for medico-legal purposes in the identification and differentiation of blood stains and in my experience has proven far more sensitive and decisive in its results than the precipitin test (see Chapter L).

Or if a sausage were suspected of containing dog flesh, an extract may be employed with known antidog serum and under suitable conditions the adulteration thereby detected.

Complement-fixation tests of either kind can be rendered highly specific and very sensitive by proper attention to technical details and have proven of great value in serum diagnosis, as I hope to make plain in succeeding chapters.

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CHAPTER IV.

NATURE AND PROPERTIES OF COMPLEMENT-FIXING ANTIBODY WITH SPECIAL REFERENCE TO THE WASSERMANN REACTION.

No one has ever seriously questioned the role of antibody in the mechanism of the specific complement-fixation reaction and it may be well to discuss its nature and properties at the present time. But several have questioned the role of antibody in the mechanism of the Wassermann reaction and it is first necessary to consider this important question.

COMPLEMENT-FIXING ANTIBODY AND THE WASSERMANN REACTION.

When Wassermann and his colleagues and Detre first described complement-fixation in experimental and human syphilis in 1905, no one questioned the assumption that it was due to the fixation of complement by syphilis antibody and *Spirochæta pallida* contained in the extracts of syphilitic tissues employed as antigens. But when it was subsequently proven that excellent and satisfactory "antigens" could be prepared of watery or alcoholic extracts of non-syphilitic tissues, several investigators abandoned the idea of its being an antibody reaction and offered several theories aiming to show that the mechanism was one involving quantitative or qualitative changes in the globulins of serum or spinal fluid, the access of tissue disintegrative substances in the nature of amino-acids, changes in the lipoids, changes in the physical or physico-chemical properties of serum, etc. Neisser spoke of the antibody-like substance as a "reagine," referring to the presence of some substance produced by the action of *Spirochæta pallida* upon tissue cells but not in the nature of a true antibody. For many years I accepted this idea and term but I now believe that sufficient evidence has been produced to indicate that the Wassermann reaction is produced by an antibody of the same kind as responsible for other complement-fixation reactions, although the source and production of this antibody is still obscure, as reviewed in Chapter VI.

The question would be definitely settled if any one succeeded in isolating the Wassermann substance from syphilitic serum. Wassermann[1] and Citron[2] indeed claim to have accomplished this by

dissolving the precipitate formed between syphilitic serum and lipoidal tissue extracts in 1.7 per cent sodium chloride solution followed by filtration through a colloid filter like the deHaen membrane or by passing through powdered infusorial silica. They state that the isolated antibody fixed complement with ordinary extracts and otherwise acted like an antibody (amboceptor). Prausnitz and Stern[3] however, failed to confirm these results and Epstein[4] has likewise questioned the correctness of the hypothesis.

In my experience the maximum degree of precipitation in syphilitic serum occurs when serum and Kahn extract are brought together in the proportions and manner so carefully worked out by Dr. Kahn in developing his serum precipitation test. Since this extract also yields good complement-fixation reactions in syphilis when properly titrated and used in the correct amount, I had Dr. Uchida try to dissociate the complement-fixing substance from the precipitates, but his attempts were not successful although he succeeded in dissociating small amounts of the typhoid complement-fixing antibody from sensitized bacilli.

In additional experiments Miss Yagle and I have been scarcely more successful in dissociating small amounts of complement-fixing antibody from the precipitates extracted with 1.7 per cent sodium chloride solutions. For example in one experiment we added 20 cc. of diluted Kahn extract to 5 cc. amounts of strongly Wassermann-positive serum and after incubating the mixtures in a water-bath for one hour at 38° C. placed them in a refrigerator over night for maximum precipitation. The supernatant sera were now removed, the precipitates washed with cold saline solution and one extracted with 5 cc. of 10 per cent saccharose in water, a second with 5 cc. of distilled water (later rendered isotonic) and a third with 2.5 cc. of 1.7 per cent sodium chloride (to which 2.5 cc. of distilled water were subsequently added to restore isotonicity). These extracts were thoroughly centrifuged to remove particles of precipitate, best results being observed with the saccharose and saline preparations, heated at 55° C. for fifteen minutes and tested in amounts of 1 cc., 0.5 cc., 0.25 cc., etc., according to the technique of my method described in Chapter XXXI. Each was tested at the same time for anticomplementary effects (without antigen) in the same amounts.

The saccharose extract was very slightly anticomplementary in dose of 0.06 cc. but gave no complement-fixation in the same dose. The distilled water extract was very slightly anticomplementary in dose of 0.06 cc. but likewise gave no appreciable degree of complement-fixation reaction in the presence of antigen in this amount. The 1.7 per cent sodium chloride solution however, after dilution with an equal amount of distilled water, was not anticomplementary in dose of 0.06 cc. but gave a complement-fixation reaction in this amount.

Taoka having proposed extraction with ether as a method for dissociating antibody and antigen, Miss Yagle and I added 3.6 cc. of diluted Kahn antigen to 6 cc. of syphilitic serum and after water-bath incubation for fifteen minutes followed by overnight in the refrigerator, secured the precipitate by centrifuging, washed it with saline solution and extracted with 6 to 8 cc. of ether. The precipitate suspended in 6 cc. of saline solution gave strongly positive Wassermann reactions, presumably due to the presence of dissociated antibody in the suspension; the ether extract diluted 1 to 100 with saline acted as an efficient antigen, presumably due to dissociated antigen.

In a duplicate experiment the saccharose and water extracts again failed to show any appreciable degree of dissociation of complement-fixing antibody but the saline extract which was not anticomplementary in dose of 0.25 cc. gave a weak fixation in this amount. It would appear therefore, that when precipitates are produced with *very strongly positive sera* that some complement-fixing antibody may be dissociated in 1.7 per cent saline solution and that it is responsible for the Wassermann reaction conducted with alcoholic extracts of beef heart as "antigen." As I shall discuss shortly it would appear that this complement-fixing antibody is identical with the precipitin or closely related to it. Only the production of the Wassermann antibody would appear to be obscure, that is, how and why an antibody is produced in syphilis capable of reacting with the lipoids of non-syphilitic tissues like beef heart but I believe that this may be explained by the data indicating that it is heterophil in nature.

CHEMICAL NATURE OF COMPLEMENT-FIXING ANTIBODY.

The chemistry of immune sera containing complement-fixing antibodies is of considerable interest not only in relation to the exact chemical nature of antibodies in general and that concerned in complement-fixation in particular, but as previously stated, because some maintain that the Wassermann reaction is not due to an antibody at all but to an alteration in the quantity or quality of the globulins or lipoids of serum. But I believe that an antibody is concerned in the Wassermann reaction and that it, as well as the antibodies responsible for specific complement-fixation reactions, is of a protein constitution belonging to the globulin fractions of both serum and spinal fluid.

In the first place Rowe,[5] employing a refractometric method, has observed an increase of the globulins of the serum in syphilis and Tokuda,[6] whose work I have been able to follow closely, confirmed these observations, as likewise have Schiff and Roser,[7] Bircher and McFarland,[8] Schmidt[9] and others. Of course this increase of glo-

bulins in the plasma of syphilitics does not necessarily indicate that the complement-fixing antibody in this disease belongs to the globulins or that the Wassermann reaction is due to an increase or alteration of them; indeed, the antibody may be present without a detectable increase of the globulins but by means of "salting out" and other methods, Kapsenberg[10] found the syphilis complement-fixing antibody in the globulin fraction of serum and Stern[11] has further identified it with the euglobulins as likewise has Harold[12] working with the spinal fluids of syphilitics. Veller and Schierge[13] however, commenting upon the difficulty and uncertainty of fractionating methods, state that in syphilis all of the proteins are changed and not only the globulins; Kaffka[14] states the Wassermann reaction in spinal fluid does not depend upon the presence of globulins, nor upon an increase of them or an alteration of their physical state. But it is well known that the proteins and especially the globulins are so uniformly increased in the cerebrospinal fluid of syphilis that its routine examination in this disease always includes one or more of the simpler tests for them.

The very careful work of Gilmour[15] with the sera of syphilitic individuals has shown that the antibody or other substance responsible for the Wassermann reaction is identified with the globulins and more particularly with the pseudo-globulin fraction. During the past year Uchida,[16] working in my laboratory, has succeeded in isolating the typhoid complement-fixing antibody in quite a pure state by dissociating it from sensitized bacilli by means of sugar solutions followed by further purification, the methods being similar to those employed by Huntoon and his associates in the isolation of the pneumococcus antibody. Such solutions did not sensitize guinea-pigs but yielded some of the protein reactions, the general results suggesting that the antibody was a globulin. Of interest in this connection are likewise the observations of Schuringer and Kapsenberg[17] and of Kendrick and Kahn,[18] indicating that the antibody concerned in the Sachs-Georgi and Kahn precipitation reactions in syphilis is carried down in the globulin fractions of serum after 50 per cent saturation with ammonium sulphate. I mention these observations here in relation to the chemical nature of the complement-fixing antibody because I believe that the same antibody is responsible for both reactions or, in other words, that precipitin and complement-fixing antibody are either one substance or very closely related, not only in syphilis, but in other diseases as well, a subject to be considered shortly with more detail. But whether or not one is justified in concluding that the complement-fixing antibody in all diseases is identified with the globulins, as is apparently the case in syphilis, is an open question. Pinner[19] for example, has recently reported that in tuberculosis it is not in the nature of a globulin at all but associated either with the lipoids

or those proteins of the serum which are not broken up by the action of trypsin.

Certainly the complement-fixing antibody, including that concerned in the Wassermann reaction, does not appear to be ether soluble or otherwise identified with the lipoids of serum and cerebrospinal fluid. Peiitz[20] states that the serum lipoids are increased in syphilis but Bauer and Skutezky[21] observed no parallelism between the lipoid and antibody content. Uchida, referred to above, found that the isolated typhoid complement-fixing antibody was not ether soluble and von Wassermann[22] has made a similar statement in reference to the antibody in syphilis which, shortly before his death, he claimed to have isolated. Forssmann[23] also states that the syphilis antibody is not ether soluble, and both he and Friedmann[24] incline to the view that the syphilis complement-fixing antibody is either a lipoid associated with globulin or a globulin-soap compound. Several investigators have observed that there is no parallelism between the Wassermann reaction and the cholesterol content of sera in syphilis.

Others like Bachman[25] believe that the antibody responsible for the syphilis reaction is a product of tissue disintegration because it was found that the ninhydrin test for free amino-acids in the protein-free residue of serum ran parallel with the strength of the Wassermann test and because the addition of minute amounts of glycocoll, leucin or tyrosin to Wassermann-negative sera resulted in producing positive reactions. In my experiments however, these results could nor be confirmed and Forssmann[26] likewise reached the conclusion that the syphilis antibody could not be an amino-acid; furthermore, I have not been able to produce the syphilis complement-fixing antibody in Wassermann-negative rabbits by injections of amino-acids, as claimed both by Bachmann and Much and Schmidt.[27]

I believe therefore, that the complement-fixing antibody concerned in both the specific and non-specific reactions including the Wassermann reaction, is a globulin in chemical structure and very probably a pseudo-globulin. Furthermore, the substance responsible for the non-specific complement-fixation reactions yielded by normal rabbit, dog and mule sera would appear to be a globulin, as shown by Trist and myself[28] several years ago. Even the anticomplementary substances of serum would likewise appear to be identified with the globulins, as recently shown by Uchida[29] in my laboratory and to which further reference will be made in Chapter VII.

SOURCE OF THE COMPLEMENT-FIXING ANTIBODY.

But little can be written of the source of complement-fixing antibody, that is, the tissues concerned in its production either during infection or as a result of artificial immunization, because our inform-

ation is so incomplete upon the question of source of production of all antibodies in general. It is highly probable however, that the lymphoid tissues participate actively in the process and likewise the vascular endothelium.

Wassermann and Lange have expressed the opinion that the antibody concerned in the Wassermann reaction may be produced by small lymphocytes because they have secured them by centrifuging syphilitic spinal fluids and observed that when the cells were resuspended in saline solution and heated for prolonged periods at 57° C. the fluid reacted positively whereas similar preparations of cells from cases of meningitis did not yield the complement-fixing substance. On several occasions I have repeated this experiment with cells from the cerebrospinal fluids of paretics and usually with weakly positive results suggesting that minute amounts of antibody may be obtainable by the autolysis of these cells. Furthermore, Klauder and myself[30] have repeatedly observed that the secretions of chancres yield positive Wassermann reactions when none of the antibody could be detected in the blood serum; this suggests that it may be produced in the chancre by the cells directly stimulated by *Spirochæta pallida* and its toxins although we could not exclude the possibility of the negative blood serum reactions being due to high dilution of the antibody in the blood. But insofar as the Wassermann reaction is concerned I think it is highly probable that the antibody is produced by the lymphocytes and other cells in the areas of acute and chronic syphilitic infection and that antibody production may occur therefore, in any tissue or organ where *Spirochæta pallida* happens to lodge. I believe this is one reason why cerebrospinal fluid may sometimes yield a positive Wassermann reaction when the serum reacts negatively, that is, because in such cases there is more antibody production in the syphilitic deposits in the meninges than elsewhere in the body resulting therefore in a higher concentration in the cerebrospinal fluid.

PROPERTIES OF THE COMPLEMENT-FIXING ANTIBODY.

Most of our information concerning the physical properties of the complement-fixing antibodies has been gained by a study of them in serum. Uchida, whose work has been previously referred to in this chapter, has succeeded however, in isolating small amounts of the typhoid complement-fixing antibody in a high state of purity for studies on its chemistry, resistance to heat, tryptic digestion, filterability, etc.

The complement-fixing antibodies are ordinarily highly resistant to deleterious influences. Probably the most important property is resistance to *heat* because of the custom of heating sera at 55° C. preliminary to testing. Sera sometimes contain small amounts of

natural or native complement-fixing antibodies and these are usually more susceptible to heat than those produced during disease or artificial immunization. At 55° C. some antibody is destroyed and especially if the exposure is longer than fifteen minutes, a subject considered with more detail in Chapter XIX because of its great practical importance. At 60° to 62° C. a good deal more antibody is lost if the exposure is maintained for more than thirty minutes while temperatures of 70° to 100° C. rapidly and completely destroy all complement-fixing antibodies. Uchida found that the isolated typhoid complement-fixing antibody was uninjuried at 55° C. for thirty minutes but underwent slight deterioration at 60° C., marked deterioration at 70° C. and complete destruction at 80° C.

These antibodies however, are preserved for long periods at low temperatures. *Freezing* apparently causes no deterioration at all although alternate freezing and thawing results in gradual destruction. I have kept syphilitic sera at or near 0° C. for six years with apparently no loss of antibody.

They are likewise highly resistant to *desiccation* and especially if immune sera are dried rapidly and at low temperatures. In this manner syphilitic and other sera containing gonococcus, typhoid, tuberculosis, glanders and such complement-fixing antibodies may be preserved in powder form for indefinite periods of time with little or no deterioration after the initial loss of antibody at the time of drying.

They are likewise highly resistant to such *chemical agents* as phenol, formalin and other preservatives. They apparently resist destruction by putrefactive changes in serum for some time although, as observed by Uchida[31] in my laboratory, these changes render sera highly anticomplementary and may bring about a gradual destruction of the Wassermann antibody at the same time. But when immune sera are kept in a sterile condition at temperatures of 0° to 10° C. the complement-fixing antibodies are preserved for months and even years with little or no appreciable deterioration, indicating that they are highly resistant to *age*.

As shown by Sachs and Altmann[32] the syphilis complement-fixing antibody is quite susceptible to destruction by *acids* and *alkalies;* sodium hydroxide was found to be less destructive than hydrochloric acid. By adding the acid to syphilitic serum it was possible for them to destroy its reacting power, so that when the mixture was, later on, neutralized and "antigen" added, complement fixation failed to occur. Abramow[33] observed similar results and the subject is considered with more detail in Chapter VIII because of its practical importance in relation to the influence of changes in hydrogen-ion concentration, the preparation of glassware, etc. According to Friedberger and Shiga[34] the prolonged exposure of syphilitic serum to *radium* weakens the Wassermann reaction; the

exposure of alcoholic extract of syphilitic liver rendered the extract inactive but cholesterol was found to protect against this influence. Brann[35] reports that *roentgen-rays, radium* and *ultra-violet light* have practically no influence upon the properties of Wassermann-positive and negative sera although complement is slightly susceptible.

Complement-fixing antibodies are freely *dialyzable*. Miss Yagle and I found that the Wassermann antibody is freely dialyzable although the serum sometimes acquires anticomplementary properties in the process. Tests with 33 syphilitic sera showed practically the same reactions after as before dialysis through parchment shells; Uchida likewise found dissociated typhoid antibody to be dialyzable.

Filterability depends upon the kind of filter employed. Ordinarily filtration through sterile, neutral Berkefeld and Mandler filters removes some Wassermann and other complement-fixing antibodies especially if the sera are diluted and the first few cubic centimeters of the filtrates are tested. Filtration through collodion sacs and porcelain filters generally removes all or most of the syphilis antibody.

Adsorption of syphilitic and other immune sera with such substances as barium, kaolin and charcoal does not ordinarily remove complement-fixing antibody although some sera may be rendered anticomplementary.

IMMUNOLOGICAL NATURE OF COMPLEMENT-FIXING ANTIBODY.

This now brings one to a discussion of the immunological nature of the complement-fixing antibody, that is, its relation to other antibodies and especially to the precipitins and agglutinins.

This at once involves the question of the unity of antibodies so ably championed by Friedberger, Zinsser, Dean and Nicolle, but I shall not review here the arguments for and against the hypothesis since these have been recently presented in a complete manner by Wells[36]. But I have conducted considerable investigation upon the subject insofar as it concerns complement-fixation and the results have convinced me that precipitins and complement-fixing antibodies are either identical or so closely associated in the phenomenon of complement-fixation as to demand the presence of both for the reaction.

In his work upon the isolation and purification of the typhoid complement-fixing antibody, Uchida almost invariably found that agglutinin was likewise present in the solutions and in a general manner the amount of complement-fixing antibody was roughly parallel to the agglutinin content of the many different solutions prepared by him. In additional experiments I have observed that solutions of dissociated typhoid complement-fixing antibody also contained precipitin for the dissolved bacilli, although the results of

these experiments were not as clear-cut as Uchida's agglutination reactions because of the greater technical difficulty of attaining the optimum concentrations of antigen and antibody for the reactions.

Dean[37] has shown in quite a convincing manner that serum precipitins and complement-fixing antibodies are either identical or both required for complement-fixation. Miss Yagle and I have observed that the removal of precipitin from antisheep serum by the addition of sheep serum followed by centrifuging to remove the precipitate always resulted in the removal of complement-fixing antibody for sheep serum antigen; in additional experiments with antihuman, antihorse, antidog, and antiox sera, similar results have been observed.

Similar results have been also observed with syphilitic sera. By adding the proper amounts of Kahn antigen to secure the maximum degree of precipitation followed by a removal of the precipitates, we have found that the complement-fixing antibody was likewise removed at the same time. Seki[38] has reported similar results although in his experiments the treatment of syphilitic serum with *Spirochæta pallida* in emulsions of syphilitic liver failed to remove the Wassermann antibody. Stuhmer and Merzweiler[39] treated syphilitic sera with the Sachs-Georgi extract and found that precipitation removed the complement-fixing antibody. We have likewise employed the Sachs-Georgi and cholesterolized alcoholic extracts of beef heart (employed in the Wassermann test) and observed similar results, although best results were secured with the Kahn antigen diluted and added to syphilitic sera in the proportions so carefully worked out by Kahn for securing the maximum degrees of precipitation. Breinl[40] has absorbed syphilitic sera with powdered beef heart, reporting that the sera could be rendered Sachs-Georgi- and Wassermann-negative; our experiments with finely divided beef heart powdered resulted however, in only a very slight removal of precipitin (for Kahn antigen) and complement-fixing antibody.

Sometimes the precipitates secured with Kahn antigen were found to fix complement when resuspended in saline, heated at 55° C. for fifteen minutes and tested. The fluids were likewise sometimes rendered anticomplementary by the process of precipitation but a sufficient number of tests have been conducted to convince me that the precipitin and complement-fixing antibodies in syphilitic and other immune sera are removed together in the precipitates and are either identical or so closely related that both are necessary for the phenomenon of complement-fixation. The mere fact that the Kahn and Wassermann reactions agree in at least 90 per cent of syphilitic sera insofar as positive or negative reactions are concerned, furnishes additional presumptive evidence of the possible unity of the antibodies. Absolute parallelism between precipita-

tion and complement-fixation however, cannot be expected in the present state of our imperfect knowledge of the mechanism of the reactions and consequently the imperfections of methods; both probably depend upon the presence and concentration of electrolytes, the pH, the mutual relations of concentration, viscosity and other physico-chemical factors. As Dean has pointed out, the reason why the two reactions do not run a parallel course is because they represent two phases of the same reaction, and it may not be possible to demonstrate both under the same conditions.

But it may be argued that during precipitation the complement-fixing antibody is mechanically carried down in the precipitate; indeed Noguchi[41] has reported that when antihuman serum is added to syphilitic serum, the production of a precipitate may rob it of the capacity for producing a positive Wassermann reaction.

The results of my experiments however, do not indicate this possibility. For example, I have found that a syphilitic serum containing typhoid agglutinin and complement-fixing antibody treated with Kahn antigen followed by removal of the precipitate, does not result in the removal of the typhoid antibodies, although both the syphilitic precipitin and complement-fixing antibody were removed. I have likewise added 0.2 cc. of immune antihuman precipitin serum to 2 cc. amounts of 1 to 50 dilutions of several syphilitic sera and, after removal of the heavy precipitates and heating of the fluids at 55° C., found little or no removal of syphilis antibody as compared with the controls although the process of precipitation frequently rendered the sera slightly anticomplementary which interfered with the interpretation of results. I have also observed that syphilitic sera containing antisheep hemolysin, treated with Kahn antigen, showed the removal of the syphilis antibodies but not of the hemolysin although in some experiments the fluids were less hemolytic because they were rendered anticomplementary and thereby masked the presence of the hemolysin. Browning and Wilson[42] have shown that if globin is added to any serum a precipitate is formed which does not fix complement except in the case that the serum is an antiglobin immune serum, when specific complement fixation takes place. I believe the results of these experiments show therefore, that during precipitation the coincident removal of complement-fixing antibody is not a mere matter of physical adsorption by the precipitate; rather they indicate that the precipitin and complement-fixing antibodies are identical or so closely related that both are removable by absorption by the same antigen.

Furthermore, it would appear that bacterial agglutinins and complement-fixing antibodies are either identical or closely related; at least the removal of the agglutinins appears to remove complement-fixing antibody in equal degree. Felix and Olitzki[43] have found that the complement-fixing antibody produced by typhoid and paratyphoid bacilli is apparently identical not only with the

small-flaking or stabilotropic agglutinins but likewise with the bactericidal antibody as well.

In my experiments a rabbit antityphoid serum gave an agglutinin titer of 1 to 640 and a complement-fixing titer of 1 to 1200. After extracting a portion of the serum with typhoid bacilli and removing the sediment of agglutinated organisms, the supernatant fluid gave an agglutinin titer of 1 to 25 and a complement-fixing titer of 1 to 280. An additional extraction with bacilli removed all of the agglutinin and apparently all of the complement-fixing antibody, although I could not be absolutely sure of the latter because the fluid was slightly anticomplementary. Repeat tests with a second antityphoid serum and two glanders sera from infected horses yielded similar results.

So I believe the available evidence indicates that the precipitins, agglutinins and complement-fixing antibodies are either identical or very closely related. Conclusive evidence of their unity has not been produced and the removal of precipitin or agglutinin or both along with complement-fixing antibody by absorbing a serum containing them with antigen, does not prove their unity because the one antigen could absorb and thereby remove several antibodies at the same time.

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CHAPTER V.

NATURE AND PROPERTIES OF ANTIGENS IN RELATION TO COMPLEMENT FIXATION WITH SPECIAL REFERENCE TO THE WASSERMANN REACTION.

The subject of antigens in relation to complement-fixation constitutes one of the most important phases of the subject.

By definition antigens are substances which cause the production of specific or non-specific antibodies. They may be injected into the tissues or blood of human beings or lower animals and antibodies thereby produced by artificial immunization or they may arise during disease. In the diseases caused by bacteria and other parasites the organisms constitute the antigens and the various antitoxins, opsonins, agglutinins, alexofixagins, etc., are the different antibodies. But in relation to complement-fixation it is known that different substances vary greatly in their ability to engender the production of antibodies and it is important to inquire into this subject in relation to the production of complement-fixing antibody since this concerns so vitally the sensitiveness and practical value of the complement-fixation test in serum diagnosis.

By common usage, however, the word antigen has been likewise applied to the substance employed in the test-tube for interaction with the antibody. And this is correct in the great majority of immunological reactions because the substance causing the production of the antibody also reacts with it *in vitro*. But there are exceptions to this rule. For example the protein-free lipoids are not believed capable of causing the production of antibodies but yet it is definitely proven that they may interact with them in the test-tube. This is strikingly shown in the Wassermann and precipitation reactions in syphilis in which tissue and other lipoids react with the syphilis antibody. In other words as stated by Hadjapoulos and Burbank,[1] antigens may be composed of two dissimilar molecules: (*a*) the *immunogenic* or antibody-producing fraction and (*b*) an *immunophilic* or antibody-reacting fraction.

Therefore, it is necessary to discuss the subject of antigens in relation to complement-fixation from two important but distinct angles, namely, (*a*) in relation to the production of antibody and (*b*) in relation to interaction with the antibody *in vitro* in the conduct of complement-fixation tests.

ANTIGENS IN RELATION TO THE PRODUCTION OF COMPLEMENT-FIXING ANTIBODY.

It is highly probable that the following three factors (named in the order of importance) influence the occurrence and sensitiveness of complement-fixation reactions in disease: (*a*) The *extent and location of the infection and virulence of the microparasite* as influencing the degree of stimulation of the antibody-producing tissues; (*b*) the *nature* of the infection, that is, *the kind of microparasite* producing disease and (*c*) the *technique of the complement-fixation test* as influencing the amount of antibody detectable in the blood and other body fluids.

Undoubtedly the location and severity of an infection regardless of the microparasite is the most important factor as shown in gonococcus and tuberculous infections, but aside from this factor evidence summarized below indicates that *the same numbers of different bacteria vary in degree of stimulation of production of complement-fixing antibody.*

Variation in the Production of Complement-fixing Antibody by Microparasites.—Unquestionably the largest amounts of complement-fixing antibody are produced in syphilis and frambesia tropica of man caused, respectively, by *Spirochæta pallida* and *Spirochæta pertenuis*. These two closely related organisms would appear to be more antigenic than any other organism, insofar as the production of complement-fixing antibody in human beings is concerned. Possibly other spiral-shaped parasites like *Spirochæta icterohæmorrhagica* (infectious jaundice), *Spirochæta gallinarum* (hen spirillosis), *Spirillum obermeirei* (European relapsing fever), etc., are likewise antigenic but the evidence has not been produced.

It is also likely that *living organisms are more antigenic than heat or chemically killed organisms.* When rabbits were injected with approximately equal numbers of living and heat-killed suspensions of *Spirochæta pallida* from syphilitic testicles of rabbits and pure cultures per kilogram of weight, I found that the former appeared to produce slightly more antibody than the latter and the same was observed in duplicate experiments employing living and heat-killed typhoid and glanders bacilli. But the maximum of antibody production appears to occur during actual disease. For example, rabbits injected intravenously with living and heat-killed suspensions of *Spirochæta pallida* have not in my experience developed as much antibody as those which have been infected testicularly and presenting the chronic lesions of syphilis. Immunization of human subjects with heat-killed typhoid vaccine does not result in as much antibody production as develops during typhoid fever. Of course the chief factor of variation may be the numerical strength of the antigen and the time factor, but it is highly probable that all organisms and notably *Spirochæta pallida* and *B. tuberculosis*, are most

antigenic in the living state and by the production of the actual lesions of disease.

The bacteria vary greatly in the production of complement-fixing antibody; at least this has been found true in the case of rabbits. With the assistance of Miss Trist, I have given these animals eight weekly intravenous injections of heat-killed suspension containing 500,000,000 per cubic centimeter, beginning with 0.05 cc. and ending with 0.4 cc. *per kilogram of weight* of the following organisms; *B. mallei; B. typhosus; B. tuberculosis* (human); *B. diptheriæ B. anthracis; B. subtilis; gonococcus; meningococcus* (normal); *streptococcus* (hemolytic); *pneumococcus* (Type I) and *staphylococcus pyogenes aureus*. These were selected as representatives of Gram-positive and negative bacilli and cocci, spore and non-sporing bacilli and an acid-fast bacillus.

Preliminary macroscopic agglutination and complement-fixation tests were conducted and repeated at intervals throughout the eight weeks of immunization and finally about ten days after the last injections. The results however, may be summarized in only a broad and general manner because despite the use of a large number of animals for each microörganism *the factor of individual variation in antibody response on the part of different rabbits receiving injections of the same antigen in exactly the same dose per body weight was such that too close deductions were not permissible.*

The results of agglutination and complement-fixation tests shown in Table 1 are taken from a series conducted with the respective immune sera four days after the sixth injection of the different antigens, and are fair examples of the results observed, which may be summarized as follows with particular reference to the production of complement-fixing antibody:

TABLE 1.—VARIATION IN THE PRODUCTION OF AGGLUTININS AND COMPLEMENT-FIXING ANTIBODY BY RABBITS IMMUNIZED WITH THE SAME NUMBERS OF DIFFERENT BACTERIA ACCORDING TO BODY WEIGHT. RESULTS AFTER SIX INJECTIONS.

Microörganisms.	Agglutination*	Complement-fixation†		Increase in fixing activity.
		Before immunization, cc.	After immunization, cc.	
B. mallei	1 to 960	0.006	0.0001	60
B. mallei	1 to 640	0.050	0.0002	250
B. typhosus	1 to 320	0.025	0.0010	25
B. typhosus	1 to 480	0.050	0.0008	60
B. tuberculosis	1 to 100	0.025	0.0010	25
B. tuberculosis	1 to 80	0.006	0.00018	33
B. diphtheriæ	1 to 80	0.006	0.0002	30
B. anthracis† (less)	1 to 10	0.013	0.0080	2
B. subtilis‡ (less)	1 to 10	0.013	0.0130	0
Gonococcus	1 to 40	0.050	0.0030	16
Gonococcus	1 to 30	0.025	0.0015	16
Meningococcus	1 to 160	0.025	0.0008	30
Pneumococcus (Type I) less	1 to 10	0.025	0.0020	12
Streptococcus	1 to 10	0.025	0.0020	12
Staphylococcus aureus	1 to 60	0.013	0.0015	9
Staphylococcus aureus	1 to 40	0.050	0.0250	2

* Preliminary tests negative in 1 to 10 dilutions with all sera and microörganisms.

† Smallest amounts of sera (heated at 55° C. for fifteen minutes) yielding non-specific (before immunization) and specific reactions (after immunization).

‡ Immunized by intraperitoneal injections.

1. Preliminary agglutination tests showed that none of the sera of normal adult rabbits in final dilutions of 1 to 10 and higher contained demonstrable amounts of agglutinins for the eleven different kinds of microörganisms employed in this study.

2. Immune agglutinins were best produced by the Gram-negative bacilli (glanders and typhoid) and next by the Gram-negative cocci (the meningococcus and gonococcus). Next by the Gram-positive bacilli (diphtheria and tuberculosis) including an acid-fast bacillus (tuberculosis), the Gram-positive cocci (pneumococci, streptococci and staphylococci), and least by the spore-bearing bacilli (anthrax and subtilis).

3. The preliminary complement-fixation tests with sera heated at 55° C. for fifteen minutes showed that the sera of the majority of rabbits yielded non-specific reactions with the different antigens. All of those included in Table I yielded these reactions, quantitative tests being conducted and the smallest amounts of serum-yielding positive reactions being recorded and given in this table.

This property of normal rabbit serum for yielding non-specific complement-fixation reactions greatly complicated the problem; for this reason all complement-fixation tests were conducted with varying amounts of each serum to determine and measure the degree of specific complement-fixation by immune antibody.

It is worthy of special note in this connection that *differences in occurrence and degree of non-specific complement-fixation with the sera of normal rabbits and the eleven different antigens appeared to vary with both the rabbit and the kind of microörganism; in other words, bacterial antigens containing approximately the same numbers of different bacteria reacted differently with the serum of any one animal.* For this reason no one antigen can be used for the selection of positively and negatively reacting rabbits; the only sure way is to actually conduct preliminary complement-fixation tests with the particular antigen to be employed, as discussed with more detail in Chapter XXVIII.

4. *The different bacteria undoubtedly varied in the degree of stimulation of the production of complement-fixing antibodies.* As a general rule, the bacilli produced more complement-fixing antibody than the cocci and positive complement-fixation reactions are to be expected more in bacillary diseases (typhoid fever, glanders, tuberculosis, pertussis, etc.) than in coccus diseases (pneumonia, gonorrhea, streptococcus, staphylococcus infections, etc.). The Gram-negative bacilli employed (*B. mallei* and *B. typhosus*) produced most agglutinin and most complement-fixing antibody and agglutination and complement-fixation reactions are well known to be especially well defined in these diseases.

5. The Gram-positive bacilli (*B. tuberculosis* and *B. diphtheriæ*) ranked next in the production of complement-fixing antibody while

the spore bearing bacilli (*B. anthracis* and *B. subtilis*) yielded almost negative results.

6. The Gram-negative cocci (gonococcus and meningococcus) produced more complement-fixing antibody than the Gram-positive cocci (pneumococci, streptococci and staphylococci).

7. As a general rule bacteria producing most agglutinins also produced most complement-fixing antibodies.

But as previously stated only broad and general deductions are justified because it is well known that animals of the same species vary greatly in antibody production when injected with the same substances in the same amounts according to body weight. Furthermore antibody production does not run parallel with the amounts injected as very small doses sometimes produce more antibody than large ones.

The Chemistry of Antigens in Relation to the Production of Complement-fixing Antibody.—Only soluble proteins or substances containing them are definitely known to be antigenic, that is, capable of producing complement-fixing and other antibodies. Cleavage products are not antigenic or but feebly so and antigenic activity is best when the protein is complete, that is, contains its full quota of amino-acids. The isolated free amino-acids are not antigenic although when the cleavage products are reunited artificially (plasteins) some degree of antigenic activity may be restored. The nucleoproteins are apparently antigenic and as shown by Lake[2] may produce precipitin and complement-fixing antibody when failing to sensitize guinea-pigs. But these reactions are not specific for nucleoprotein and it is highly probable that the antigenic activity of so-called nucleoprotein preparations is largely due to the adventitious proteins so apt to be present in them.

Furthermore, it would appear that the protein molecule must be soluble in the tissues of the animal in order to produce antibody; for this reason boiled irreversible proteins are not antigenic although heated reversible proteins may produce antibody somewhat specific for its heated antigen *in vitro*, as will be discussed with more detail in Chapter XXXVII on the Detection of Cooked Meat Adulteration by the Complement-fixation Test.

It is not definitely known which radicals of the protein molecule are most antigenic but since gelatin is non-antigenic and is lacking in aromatic radicals, it has been suggested that these radicals are of importance in relation to antibody production.

The bacterial toxins are usually highly antigenic in the production of antitoxins but produce smaller amounts of precipitins and complement-fixing antibodies. Their chemistry is still undecided although it is highly probable that they are protein substances to which is attached a toxic radical, which may or may not be an integral part of the protein molecule.

But some protein-free substances may act as "antigens" in the test-tube, that is, yield precipitin and complement-fixation reactions even though they are apparently unable to produce antibody when injected into the lower animals. Substances of this kind have been apparently isolated from the typhoid bacillus, pneumococcus, other bacteria and yeasts by Pick,[3] Zinsser and Parker,[4] Heidelberger and Avery,[5] Tomcsik[6] and others. Their exact nature is unknown but they resemble complex carbohydrates in some respects and their non-antigenic activity *in vivo*, while capable of reacting *in vitro*, is analogous to the situation in regard to the protein-free lipoids. But it would appear that some of the complex glucosides which can be hydrolyzed by enzymes may produce antibodies and this may account for the production of specific antitoxins in rabbits injected with extracts of *Amanita phalloides* and *Rhus toxicodendron* by Ford.[7] But insofar as the production of complement-fixing antibody in rabbits is concerned, the antigenic activity of the glucosides has been very feeble or entirely absent in my experience since repeated intraperitoneal and intravenous injections of salicin, arbutin, safonin, digitalin, ouabain, extracts of *rhus toxicodendron*, etc., have uniformly failed to produce demonstrable amounts.

A great deal of investigation has been devoted to the question of the antigenic activity of the lipoids and I believe the following deductions are warrantable: (*a*) That protein-free lipoids do not produce antibodies in the lower animals; (*b*) that lipoid-protein substances may be antigenic and produce precipitins and complement-fixing antibody and (*c*) both protein-free lipoids and lipoid-protein complexes are capable of acting as so-called "antigens" in the test-tube yielding precipitin and complement-fixation reactions. But a great deal of confusion has been produced by work with impure lipoids and by concluding that since lipoids may act as "antigens" *in vitro* (as in the Wassermann reaction) that they are capable of producing antibody *in vivo*, that is, of acting as true antigens. In the first place as shown by Levene[8] and others, it would appear that the lipoids from the tissues of different animals are chemically the same and it is a well-established law of immunology that a substance must be foreign to the animal injected before antibody production takes place; therefore as shown by Thiele and Embleton,[9] one cannot expect the *pure* lipoids *alone* to exhibit immunological specificity.

It is hardly necessary to review here the large literature upon the subject. The majority of investigators have found pure fats and lipoids devoid of antigenic activity although a few maintain that they are not only antigenic but even of more importance in this regard than the proteins themselves. Much[10] for example, has gone so far as to state that the proteins owe their antigenic activity to admixed lipins and Warden[11] states that the true fats and salts

of the fatty acids from bacteria and other substances in a proper state of emulsification are not only capable of yielding complement-fixation reactions *in vitro* but are likewise more important and more specific than the proteins in the production of antibody *in vivo*. But while the lipoids readily take up proteins and lipoid-protein complexes are undoubtedly capable of producing complement-fixing antibody, yet it is the consensus of opinion that protein-free fats and lipoids are apparently without this power.

The Production of Complement-fixing Antibody in Syphilis.—However, since the outstanding and clearly proven quality of the syphilis antibody is the precipitation of lipoids or lipoid-protein complexes from syphilitic or non-syphilitic tissues in the test-tube followed by the fixation of complement in the Wassermann test, it may be well to inquire a little further into the production of this antibody during syphilis and yaws.

Many years ago Weil and Braun[12] suggested that during syphilis, tissue products, such as protein-lipoid complexes are absorbed, and produce the antibody. Citron[13] also suggested a similar theory in that the toxins of *Spirochæta pallida* united with the tissue lipoids forming a toxolipoid responsible for producing the antibody and Sachs, A. Klopstock and Weil[14] have more recently revived interest in the theory that the Wassermann reaction is caused by a change in the serum due to the action of a combination of lipoids of the tissues with proteins of the spirochetes and were able to show that rabbits injected with alcoholic extracts of rabbit organs along with pig serum became Wassermann-positive. However, these views have failed to find wide acceptance although Wassermann[15] has more recently renewed interest in the subject by expressing himself strongly in support of the theory that the lipoids of *Spirochæta pallida* may produce the antibody.

In the first place it may be asked whether the injection of the lower animals with *Spirochæta pallida* results in the production of complement-fixing antibody. Noguchi[16] after succeeding in cultivating this parasite in 1912 injected rabbits and found them to produce a complement-fixing antibody which he thought was specific for the proteins of the organisms in addition to the production of the substance responsible for the Wassermann reaction conducted with the ordinary alcoholic tissue extracts. Williams, Laubaugh and myself,[17] F. Klopstock[18] and others have likewise found that immunization of rabbits with pure cultures of spirochetes, believed to be *Spirochæta pallida*, resulted in the production of an antibody capable of yielding complement-fixation reactions with culture of the organism and ordinary Wassermann extracts. But the amount of antibody production is relatively small and while the reactions *in vitro* may be with the proteins of the spirochætes yet our work suggested that they may have been due to interaction

with the lipoids or lipoid-protein complexes of the organisms and analogous to reactions with the lipoid substances contained in ordinary tissue extracts. If this were true, and I believe it is, one need admit the production of but one antibody in rabbits immunized with cultures of *Spirochæta pallida*, namely the lipoidophilic antibody capable of precipitating the lipoids or lipoid-protein substances from either spirochetes or non-syphilitic tissues with the fixation of complement.

Spirochæta pallida however, appears to produce much more complement-fixing antibody in human beings than it does in rabbits; at least this has been my experience. Rabbits inoculated intratesticularly and then castrated about two weeks later or given substerilizing doses of arsphenamine in order to remove or suppress the local testicular reaction to favor dissemination of the parasites, have usually shown the presence of antibody when chronic lesions were present, but the strength of the reactions have not usually been comparable to those seen in human beings with similar lesions. Furthermore, I have injected rabbits intravenously with increasing amounts of heat-killed cultures of *Spirochæta pallida* once a week for six to ten weeks and found that their sera developed but relatively small amounts of complement-fixing antibody when tested with antigens of heat-killed culture and ordinary alcoholic tissue extracts of beef heart. In other words heat-killed cultures of *Spirochæta pallida* are but feebly antigenic for rabbits and much less so than the living organisms and these animals apparently react much less with the production of complement-fixing antibody against *Spirochæta pallida* than do human beings.

I may state in passing, however, that the complement-fixing antibody is not produced in cultures of *Spirochæta pallida*, at least not in my experience, although this is of little interest since no one has yet succeeded in producing antibodies *in vitro*.

Citron and Munk state that the injection of rabbits with watery emulsions of congenital syphilitic liver caused the animals' sera to react positively with alcoholic human heart extract as antigen; Eiken[19] corroborated their findings and Meyer[20] has more recently stated that while rabbits injected with watery extracts of syphilitic liver produced antibody, animals injected with alcoholic extracts did not although Griesbach[21] found that guinea-pigs injected with alcoholic extracts produced antibody yielding weakly positive complement-fixation reactions with these extracts but not with cholesterolized extracts of normal organs.

I have injected rabbits intravenously once a week for eight weeks with increasing amounts of the following extracts: (1) Saline and alcoholic extracts of congenital syphilitic liver; (2) alcoholic extract of beef heart; (3) alcoholic extract of beef heart plus 0.4 per cent cholesterol and (4) an extract of acetone-insoluble lipoids of beef

heart. All of these contained protein and especially the watery extract of syphilitic liver. Rabbits were selected after preliminary complement-fixation tests and only those yielding negative reactions were employed for immunization. The results have shown that the injection of these extracts sometimes produced very small amounts of complement-fixing antibody specific for the tissue (human or beef) from which the extracts were prepared. But some rabbits did not produce detectable amounts of antibody at all and in the case of those that did, I concluded that the traces of protein in the extracts were the antigenic agents and that the weakly positive reactions were due to the action of antiprotein antibody in the sera. This confirms the work of Fitzgerald and Leathes[22] and others who found that the injection of rabbits with ordinary Wassermann extracts does not ordinarily excite the production of complement-fixing antibody for them.

Of far more significance however, are the observations of Dohi[23] and especially of Taniguchi[24] who found that the injection of rabbits with watery emulsions of guinea-pig heart, liver or kidney results in the production not only of heterophil antisheep hemolysin but likewise of Wassermann antibody.

With the assistance of Miss Richter, I have prepared watery extracts of these organs in the same manner as described for the preparation of watery extracts of syphilitic liver for the Wassermann test and have given rabbits six weekly intravenous injections of increasing amounts. Preliminary tests were conducted for natural antisheep hemolysin and all rabbits showed a trace in 0.5 cc. of 1 to 5 dilutions of serum.

After immunization the hemolysin titrations were repeated and all rabbits showed a slight increase of hemolysin, the units ranging from 0.5 cc. of 1 to 10 to 1 to 40. In other words the injection of rabbits with saline extracts of guinea-pig heart, liver and kidney resulted in the production of small amounts of heterophil antisheep hemolysin.

Preliminary complement-fixation tests were conducted by my new method for rabbit, dog and mule sera (see Chapter XXXV) in which each serum was heated at 62° C. for fifteen minutes and tested in an amount of 0.01 cc. with 5 units of complement and 5 units of hemolysin to overcome or neutralize the tendency to non-specific complement-fixation; the results were as follows, the Wassermann antigen employed being my new alcoholic cholesterolized and lecithinized extract of beef heart:

Rabbit.	Heart antigen.	Liver antigen.	Kidney antigen.	Wassermann antigen.
1	−	+	+	−
2	+	+	+	−
3	−	−	−	−
4	−	−	−	−
5	+	+	+	−
6	−	+	−	−

After immunization the tests were repeated with the following results, the stronger reactions with saline extracts of guinea-pig tissues being due to the production of antiguinea-pig antibody as would be expected but those

with the Wassermann antigen being particularly interesting in relation to the possible heterophil nature of the Wassermann antibody:

Rabbit.	Injected with.	Heart antigen.	Liver antigen.	Kidney antigen.	Wassermann antigen.
1 . .	Heart extract	+	+++	++	−
2 . .	Heart extract	++	++++	+++	+
3 . .	Liver extract	+	++++	++++	++
4 . .	Liver extract	−	+++	++	+
5 . .	Kidney extract	+	+++	+++	++
6 . .	Kidney extract	−	++	++	+

The results have shown therefore, that the protein-lipoid complex in guinea-pig tissues believed to constitute the heterophil antigen for antisheep hemolysin when injected into rabbits, is also capable of sometimes producing small amounts of complement-fixing antibody similar at least to that concerned in the Wassermann reaction; this was especially true of rabbits injected with the kidney and liver extracts. In other words the results indicate the possibility of producing the Wassermann antibody in rabbits by the injection of tissue products of the guinea-pig (negative results were subsequently observed in a similar experiment with extracts of beef and human tissues) and indicates that the Wassermann antibody in the sera of human syphilitics may be heterophil in nature. Furthermore, as stated by Georgi[25] and Taniguchi[26] and more recently by Hooker,[27] it is possible that the sera of some human beings may contain this non-specific or heterophil antibody for guinea-pig tissues and thereby open up the possibility of yielding non-specific or falsely positive Wassermann reactions when extracts of guinea-pig heart are used as "antigens."

The question naturally arises whether the Wassermann antibody can be produced in human subjects by substances other than *Spirochæta pallida* and *Spirochæta pertenuis*. This concerns the specificity of the Wassermann reaction, which is discussed in detail in Chapter XL, but I may state here that an extensive experience with my new complement-fixation method has shown a very high degree of specificity for syphilis. In leprosy positive reactions may occur with other methods, suggesting that the lepra bacillus in human beings may contain a heterophil antigen for the Wassermann antibody, but I am confident that the positive Wassermann reactions reported as occurring in scarlet fever, tuberculosis, tumors, etc., have been largely due to technical errors.

Certainly the claims of Sachs and his co-workers that the Wassermann substance is the globulin of syphilitic sera altered in its state of dispersion so that it exhibits an increased precipitability in the presence of suitable organ extracts, have not been proven. Likewise

the conversion *in vitro* of unheated non-syphilitic human sera from Wassermann-negative to positive by shaking, adding agar, kaolin, bacteria, blood corpuscles, glycocoll, leucin, chloroform, ether, etc., has not been accomplished although such procedures are likely to render sera anticomplementary and thereby yield misleading results. While Much and Schmidt[28] claim to have changed the Wassermann reaction in rabbits from negative to positive by injections of pure lipoids yet Niederhoff, [29] Lange[30] and many others including myself have failed to corroborate his findings, which I believe were due to confusion because of the faculty of rabbit serum (normal or immune) for yielding non-specific Wassermann reactions.

As stated in Chapter IV, I think it proven that the Wassermann reaction is caused by an antibody. The injection of rabbits with cultures of *Spirochæta pallida* may produce it as likewise injections of extracts of syphilitic organs containing large numbers of the organisms. No other method has been found to produce it in rabbits by injection of various substances except in the case of certain trypanosomes as shown by Landsteiner, Müller and Pötzl,[31] Levaditi and Yamanouchi,[32] Browning and MacKenzie,[33] Landsteiner and van der Scheer[34] and others and possibly by extracts of guinea-pig tissues. It is highly probable that the antibody is produced by heterophil lipoid-protein antigen, contained in largest amounts in *Spirochæta pallida*, *Spirochæta pertenuis* and some trypanosomes.

ANTIGENS IN RELATION TO THE COMPLEMENT-FIXATION REACTION.

This now brings one to the second phase of the subject of antigens in relation to the complement-fixation reaction namely, a consideration of the substances capable of interaction with the antibody *in vitro* and resulting in complement-fixation. These substances may be the same as produce the antibody, in which case they are true antigens; or they may be substances unable to produce the antibody, in which event they are not true antigens. The latter may be designated as "extracts" or Landsteiner's term "haptenes" may be used; I have also designated them as "*alexofixagens*," but the word "antigen" is mostly used, is best known and probably will continue to be used even though it is a misnomer in this connection. Naturally this subject has an intimate bearing upon methods employed for the preparation of antigens for various complement-fixation tests, but in this chapter I shall consider the chemical phases of the subject and leave for Chapters XIV, XV and XVI a discussion of the technical phases.

Protein substances are excellent antigens *in vitro*. For this reason the complement-fixation test is a highly efficient means for the identification and differentiation of blood stains, sera, milks, meats,

etc.; indeed it has proven more sensitive and decisive than the precipitin reaction for these purposes.

Whole proteins are most antigenic *in vitro* just as they are *in vivo* in the production of antibody. Cleavage products and the free amino-acids are much less antigenic. But heated and even boiled albuminous substances may be antigenic *in vitro* even though they may not be capable of producing antibody, largely because of being rendered insoluble in the tissues of the injected animal. In other words albuminous substances in suspension may act as antigens *in vitro* although best results are observed with the soluble ones.

While the bacterial toxins are highly antigenic in the production of antitoxins, they usually yield but weak precipitin and complement-fixation reactions *in vitro* probably because the immune sera contain much smaller amounts of these antibodies. In my experience when horses have been immunized with the diphtheria bacillus in addition to its toxin, the immune sera have usually contained more complement-fixing antibody for the toxin used as an antigen than the sera of horses injected with toxin alone; the same has been observed in the case of horses immunized with *streptococcus scarlatinæ* and its toxin.

Non-protein substances can certainly act as "antigens" *in vitro*. This is especially true of the fats and lipoids from tissues insofar as complement-fixation in syphilis is concerned, but much less definite in the case of those substances resembling complex glucosides from bacteria, yeasts and other plants. But next to the proteins the lipoids of bacteria and other substances command most interest from the standpoint of antigenic activity *in vitro* and especially in relation to methods for preparing antigens.

The Bacterial Lipoids in Relation to Complement-fixation Reactions.— All bacteria contain some lipoid substances, notably *B. tuberculosis*, but the amount varies according to the culture medium employed and the age of the cultures. "Nastin," a lipoid material from streptothrices and tubercle bacilli, has been found by Much and others to yield positive complement-fixation reactions. But the fats and waxes of the tubercle bacillus are but feebly antigenic *in vitro* or entirely lacking in this activity as shown by Lucke,[35] similar results being observed in my experiments with these substances tested with immune calf sera and the sera of tuberculous human subjects and cattle. Caulfield and Beatty,[36] Meek,[37] Craig[38] and others have observed positive tuberculosis complement-fixation reactions with alcoholic extracts of tubercle bacilli and Much[39] with the acetone-insoluble fraction, but these extracts very probably contained proteins or protein-lipoid substances as well responsible for their antigenic activity; at least Woods, Bushnell and Maddux[40] reached this conclusion in a study of the "partial antigens" of Deyke and Much.

While it would appear therefore, that the purified lipins of the tubercle bacillus are without antigenic activity in the tuberculosis complement-fixation test, yet there is no doubt that they may react with the syphilis antibody in the Wassermann reaction and for this reason tubercle bacillus antigens may yield positive complement-fixation reactions with the sera of non-tuberculous but syphilitic individuals, unless an effort is made to remove these substances totally or in large part in the preparation of the antigens (see Chapter XV).

In my experience alcoholic, ether and acetone extracts of the gonococcus, typhoid bacillus, glanders bacillus, tubercle bacillus, and other bacteria have been so feeble in antigenic activity as to be without value in bacterial complement-fixation tests. But Warden[41] regards the protein-free alcoholic solutions of the sodium salts of the fatty acids of the gonococcus, tubercle, cholera and typhoid bacilli superior as antigens for the complement-fixation test to watery or saline suspensions or autolysates. Meyer[42] and more recently Patterson and Williams[43] have also found the acetone-insoluble lipoids, presumably phosphatids, of echinococcus cysts, "antigenic" *in vitro*, but here again my experience has not corroborated their observations, since the whole or plain cyst fluid has yielded much better reactions than plain alcoholic extracts or the acetone-insoluble fraction of the fluid and ground cysts from both human subjects and the lower animals.

In my experiments antigens were prepared of *B. tuberculosis*, *B. typhosus*, *B. diphtheriæ*, the *meningococcus*, *pneumococcus* and *Trichophyton rosaceum* by cultivation on appropriate solid media followed by removal with saline solution, washing with saline to remove traces of medium followed by drying and grounding into fine powders. Each powder was then prepared into three antigens as follows:

(*a*) 0.01 gm. was suspended in 10 cc. of saline and shaken for one hour.

(*b*) A second portion was extracted three times with 10 volume of equal parts of ether and chloroform by shaking at room temperature for four hours each time. The residues were dried, 0.01 gm. suspended in 10 cc. of saline and shaken for one hour.

(*c*) Each ether-chloroform extract was evaporated, the sticky residues dissolved in 1 cc. of ether and added to 9 cc. of alcohol which was further diluted with saline solution for the tests.

The hemolytic, anticomplementary and antigenic activities of each preparation were then determined, the last being conducted with horse or rabbit immune sera in dose of 0.002 to 0.05 cc. after heating at 60° C. for thirty minutes. The results are summarized in Table 2, the "index" of each referring to the relation between the antigenic and anticomplementary units; for example, "16" means that the antigenic unit was 16 times less than the anticomplementary unit.

As shown in the table the whole bacterial substance of each organism (antigen A) proved best; but the removal of the ether-chloroform soluble portions (antigen B) reduced the antigenic activi-

ties of all while the ether-chloroform residues (antigen C) were feebly antigenic with some and notably in the case of the tubercle bacillus. It may be therefore, that the protein-lipoid complexes of bacteria constitute the active "antigenic" substances *in vitro* in complement-fixation reactions but I believe the proteins are more antigenic than the lipoids and that the best antigens are those prepared of the whole organism rather than of any fraction.

TABLE 2.—THE INFLUENCE OF BACTERIAL LIPOIDS IN COMPLEMENT-FIXATION REACTIONS.

Organisms.	Antigen A.				Antigen B.				Antigen C.			
	Hemo.	Anticomp.	Antigenic.	Index.	Hemo.	Anticomp.	Antigenic.	Index.	Hemo.	Anticomp.	Antigenic.	Index.
B. tuberculosis	—*	1:2	1:32	16	—	1:2	1:24	12	1:2	1:8	1:64	8
B. typhosus	—	1:16	1:256	16	—	1:4	1:48	12	1:4	1:8	1:12	1.5
B. diphtheriæ	—	1:2	1:20	10	—	1:2	1:16	8	1:6	1:4	1:16	4
Meningococcus	—	1:8	1:32	4	—	1:6	1:18	3	1:4	1:8	1:8	0
Pneumococcus	—	1:2	1:8	4	—	1:2	1:6	3	1:2	1:8	1:8	0
Trichophyton	—	1:4	1:8	2	—	1:4	1:6	1.5	1:4	1:4	1:4	0

* No hemolysis with 0.5 cc. undiluted antigen.

For example, in an additional experiment employing *B. tuberculosis, B. typhosus, B. mallei* and the gonococcus, it was found that the antigens prepared of the residues of dried and ground bacteria after extraction with ether and alcohol (antigen A in Table 3) were as good as antigens of these residues plus the return of the ether-alcohol substances (antigen B in Table 3); furthermore, the addition of 0.2 per cent cholesterol to these antigens (antigen C in Table 3) did not increase their antigenic activity as it does the Wassermann extracts, but rather reduced their value by very materially increasing anticomplementary activity.

TABLE 3.—THE INFLUENCE OF ETHER-ALCOHOL SOLUBLE LIPOIDS OF BACTERIA UPON THE PROPERTIES OF ANTIGENS.

Organisms.	Antigen A*.			Antigen B.*			Antigen C.*		
	Anti-comp.	Anti-genic.	Index.	Anti-comp.	Anti-genic.	Index.	Anti-comp.	Anti-genic.	Index.
B. tuberculosis	1:4	1:30	7.5	1:8	1:60	7.5	1:32	1:64	2.0
B. typhosus	1:3	1:150	50	1:3	1:150	50	1:80	1:190	2.4
B. mallei	1:2	1:5000	2500	1:2	5000	2500	1:12	2500	200
Gonococcus	1:2	1:100	50	1:3	1:150	50	1:15	1:150	10

* No hemolysis with 0.5 cc. undiluted antigen.

In the case of antigens of tubercle bacilli I believe it advisable to remove the lipins in order to avoid cross or falsely positive reactions with the sera of syphilitic but non-tuberculous individuals; but this is not necessary in case the antigens are used for testing the sera of cattle or other of the lower animals since these do not contract syphilis. But in my experience it is not necessary to remove the lipins from other organisms like the gonococcus, typhoid bacillus, etc., in the preparation of bacterial antigens for tests with human sera because the amounts present are too small for yielding similar cross complement-fixation reactions with syphilis antibody.

The Nature of Extracts Employed as "Antigens" in the Wassermann Reaction.—It is now definitely established that the active constituents of the tissue extracts employed as "antigens" in the Wassermann test are lipoidal in nature, the term *lipoid* being employed to include not only those compounds soluble in such organic solvents as ether, alcohol, chloroform and benzol but likewise the simple fats and fatty acids. It is also definitely proven that just as efficient "antigens" may be prepared of non-syphilitic tissues as of syphilitic liver or other tissue containing *Spirochæta pallida*. Some still maintain that the latter are more efficient than the former but certainly this has not been my experience.

Naturally a great deal of investigation has been devoted to chemical analyses of watery and alcoholic extracts of syphilitic and non-syphilitic tissues to isolate and identify the constituents concerned in the Wassermann reaction. But the complexity of the preparations and the limitations of technical methods has defeated these efforts to some extent, especially in the case of watery tissue extracts, although some definite and reliable information has been gained.

In the first place vegetable lipoids may serve as "antigens" in the Wassermann reaction as shown by Walker[44] and others, but much more efficient "antigens" are prepared from tissues. The different organs of the same animal and the same organ of different species however, vary considerably in their yield of the antigenic substance. In my experience the heart muscle of cattle has proven best, but I shall discuss this subject with more detail in Chapter XIV, where the results of comparative studies are given.

The general result of extensive chemical studies in this subject and notably those of Noguchi[45] and of Browning, Cruickshank and Gilmour[46] has been to show that the chief "antigenic" substance in Wassermann "antigens" is the phosphatid lecithin and its congeners, that is, those substances soluble in alcohol and ether but insoluble in acetone (the acetone-insoluble lipoids). Pure lecithin is very difficult to prepare and in these extracts is probably associated with a variable amount of kephalin, which by itself is insoluble in alcohol. Some maintain, however, that the active constituent is a totally

different substance physically associated with the lecithin. But, as is well known, tissue extracts may be highly hemolytic, that is, capable of directly hemolyzing the test corpuscles. This is especially true of extracts of extensively autolyzed tissues and it is generally stated that the fatty acids, soaps and neutral fats are largely responsible. In the preparation of "antigen" for the Wassermann test it is necessary, therefore, to avoid the use of decomposed tissues and to get rid of these substances from the extract as much as possible, this being accomplished in my method by a preliminary extraction of the ground tissue with ether.

Tissue extracts are likewise always anticomplementary to some extent, that is, capable in themselves of fixing or inactivating complement and thereby producing inhibition of hemolysis which, under certain circumstances, may be mistaken for positive Wassermann reactions. The exact identity of these substances is not known but the proteins and protein cleavage products are believed to be important. In the preparation of "antigen" it is advisable to attempt their removal because the best extract is one almost free of anticomplementary action while retaining a high degree of "antigenic" activity. In my method this is attained by a primary extraction of the powdered beef heart muscle with alcohol, from which the highly antigenic acetone-insoluble lipoids are recovered and returned to the secondary alcoholic extract which constitutes the nucleus of the extract.

The antigenic activity of the lecithins varies greatly according to their source. The heart and liver products are usually very good while those from brain and egg-yolk are but feebly antigenic or entirely lacking in this property while being highly hemolytic.

Another out-standing result of studies in this field has been to show that while cholesterol itself is but feebly antigenic and highly anticomplementary, yet the addition of a small amount (like 0.1 to 0.2 per cent) to alcoholic solutions of the lecithins greatly increases antigenic activity. Even such small amounts as 0.014 to 0.033 per cent cholesterol added to alcoholic tissue extracts improves the "antigenic" qualities as shown by Frank,[47] but it is as yet undecided whether these effects are due to an alteration of the physical state of the watery suspension of the lecithin or to some chemical factor.

Soaps like sodium oleate, potassium stearate and sodium palmitate and the salts of the bile acids like sodium glycocholate and taurocholate and sodium cholate, are also somewhat "antigenic" and Sachs and Rondoni[48] have attempted to prepare synthetic "antigen" consisting of an alcoholic solution of lecithin, sodium oleate and oleic acid, but the results have not been nearly so good as observed with extracts of tissues; the same may be stated of Schurmann's[49] mixture of lecithin, sodium glycerophosphate, lactic acid and ammonium vanadinate. Numerous other substances like

protagon, cholin and cerebroside have been tried but with doubtful properties as "antigen" for the syphilis reaction.

Lecithin therefore, is the most important single active constituent of extracts employed as "antigen" in the syphilis complement-fixation and precipitation reactions; its "antigenic" activity is increased in both reactions by cholesterol or soaps, also by alcohol. The efficiency depends greatly upon the state of dispersion of lecithin with cholesterol in saline solution, that is, upon the size of the particles, experience indicating that the turbid emulsions of particles on the borderline in size between coarse suspensions and true colloidal dispersion, being most "antigenic." Boiling with a reflux condenser does not materially alter the properties but filtration through porcelain usually removes all "antigenic" function, as shown by Miss Trist and myself.[50]

My extract is a polyvalent alcoholic extract of powdered beef heart muscle almost entirely freed of hemolytic and anticomplementary substances and reënforced by the addition of antigenic lecithin plus 0.2 per cent cholesterol (see Chapter XIV). When titrated by my method these extracts are almost always free of hemolytic and anticomplementary properties in dose of 0.5 cc. of 1 to 8 while perfectly "antigenic" in dose of 0.5 cc. of 1 to 2000 to 1 to 4000. This leaves an enormous leeway of safety, permitting the use of 10 antigenic units in conducting diagnostic tests, this amount being usually thirty to fifty times less than the anticomplementary unit and largely accounting for the specificity of the test for syphilis.

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CHAPTER VI.

MECHANISM OF COMPLEMENT-FIXATION WITH SPECIAL REFERENCE TO THE WASSERMANN REACTION.

The exact mechanism of complement-fixation is still obscure. Numerous theories have been advanced and especially in relation to the mechanism of the Wassermann reaction,[1] but none have proven acceptable. That the reagents have colloidal properties and that the mechanism probably involves colloidal reactions is generally admitted but attempts to explain matters *solely* on the basis of the laws of physical chemistry have not met with much success.

PRECIPITATION IN RELATION TO COMPLEMENT-FIXATION.

But it has been definitely shown that precipitation may be present in both specific and non-specific complement-fixation reactions and on page 56 I have already discussed the intimate relationship between precipitin and complement-fixing antibody and their possible unity.

Twenty-one years ago Gay[2] and Moreschi[3] advanced the hypothesis that the interaction between antigen and antibody resulted in precipitation and that the precipitates adsorbed or fixed the complement; since then the general results of numerous investigations by Dean,[4] Zinsser[5] and others have suggested that precipitation may bear an essential relation to the mechanism of complement-fixation. It is true that the conditions most favorable for visible precipitation, as in the Sachs-Georgi, Kahn and similar reactions in syphilis, are not usually most favorable for complement-fixation in this disease and that the former requires a closer adjustment of the proportion of antigen and antibody, since an excess of antigen may prevent the formation of a precipitate or dissolve it, but zone reactions of this kind commonly occur in other immunological reactions.

As Dean has stated it would appear that precipitation represents the final stage of a change which can be recognized in its earliest and incomplete stage by means of the complement-fixation reaction. When antigen and antibody are mixed in the proportions most favorable for rapid and pronounced precipitation the conditions are much less favorable for complement-fixation, which occurs best

when the mixture contains smaller amounts of antigen resulting in slower and less precipitation of extremely small particles escaping naked eye detection. This is certainly true of precipitation and complement-fixation reactions in syphilis since I have found that the heavy precipitation of the Kahn reaction is accompanied by much less complement-fixation than occurs in the Wassermann reaction, in which the concentration and amount of antigen are very much less and in which macroscopic precipitation does not occur although, as shown by Jacobstahl,[6] precipitation may be sometimes detected by methods employing dark-field microscopy; indeed complement-fixation would appear to occur mainly while the precipitum is *in statu nascendi* and invisible.

But the relationship of complement-fixation to precipitation is not at all clear. Certainly one is not justified in assuming that it is a mere physical adsorption of complement by the precipitate as this fails to explain the high specificity of the reaction; rather the antibody produces a peculiar change in the antigen-antibody complex responsible for specificity which has not been adequately explained and it may be that the colloidal chemical part is to be regarded as only a preliminary step, or the final result, of a chemical process that either starts or completes the reaction and gives it specific characters.

Furthermore, it is not necessary for complement to be present at the time of precipitation in order that it shall undergo fixation. It is well known for example, that corpuscles may be sensitized in the absence of complement and that these sensitized cells fix more complement and produce hemolysis more rapidly and with less complement than when corpuscles, hemolysin and complement are brought together simultaneously. I have also observed[7] that allowing syphilitic serum and lipoidal extracts to stand at least thirty minutes before adding complement in conducting the Wassermann test sometimes increases the degree of complement-fixation. In other words affording time for antigen-antibody reaction before introducing the complement sometimes results in a stronger complement-fixation reaction than when complement is present at the outset and similar results have been more recently observed by Miss Yagle and myself in experiments with the Kahn reaction: Briefly when 2 units of undiluted guinea-pig serum complement were introduced into the Kahn system employing the antigen and syphilitic sera in exactly the same proportions as in his precipitation test, we found less fixation after incubations of (*a*) fifteen minutes at room temperature, (*b*) one hour at 38° C. and (*c*) eighteen hours at 8° C. than when complement was added to the tubes after precipitation had occurred in these three different periods of incubation. Dean has observed that if one mixes human serum and its antiserum in such proportions that visible precipitation does not

occur, the subsequent addition of whole guinea-pig serum (complement) or its euglobulin fraction may produce precipitation after an interval of six to twenty-four hours. Miss Yagle and I, however, have not observed the addition of guinea-pig serum to increase the degree of precipitation in the Kahn test, but I am referring to these observations at present to emphasize that our present knowledge of complement-fixation does not permit us to assume that it is a mere matter of physical adsorption or fixation of complement by precipitation or that complement must be present at the time of precipitation in order that its "fixation" shall occur. It is possible that important physico-chemical changes take place responsible for the effects upon complement and these may now command our attention in relation to the mechanism of both precipitation and complement-fixation.

PHYSICO-CHEMICAL FACTORS IN RELATION TO COMPLEMENT-FIXATION.

The reagents which take part in serum precipitation and complement-fixation are intimately associated with colloids and may be actually colloidal in nature. The results of excellent investigations by Walker,[8] Hirsch,[9] Hecht,[10] Kiss,[11] Coulter[12] and others leave little or no room for doubt that they are in part colloid-chemical reactions influenced by the physical state of the colloids and the pH and electrolyte content of the menstrua in which they are dispersed. But it may be stated at the outset with equal emphasis that physical chemistry has not yet explained two important phases of serum precipitation and complement-fixation namely, (*a*) the specificity of the reactions and (*b*) how complement becomes fixed or inactivated in the complement-fixation reaction.

Both precipitation and complement-fixation occur more quickly at 37° C. but proceed further at lower temperatures, which indicates their colloidal character; likewise both reactions are influenced by the size of the antigen molecules, that is, the turbidity of the diluted antigen, as recently emphasized by Silber and Friese[13] and others.

Both reactions and especially that of precipitation, require the presence of at least a minute amount of electrolyte (sodium chloride) to cause a precipitation of the electrically amphoteric colloidal complex, formed by the union of antigen and antibody, by discharging the particles unequally; Holker[14] has especially emphasized the importance of saline solution in relation to the colloidal chemistry of the Wassermann reaction, stating that the differentiation between syphilitic and non-syphilitic sera is brought out more sharply by reagents diluted with saline than with water.

The pH of the menstruum exerts some influence upon both reactions although both can take place within a wide range as recently

shown by Jennett,[15] and are not appreciably greater at the pH of either the antigen or the globulins of the immune serum with which the antibodies are associated.

According to Schmidt[16] the globulin-antibody molecules are probably electrically positive and the antigen particles electrically negative; when these are brought together in proper proportions a neutralization occurs resulting in changes suitable for colloidal precipitation. This in turn may result in an increase of hydroxyl ions as suggested by Hirsch followed by a change in the amount of electrolyte or the degree of dispersion of colloids or in some other manner reducing surface tension which, according to Hecht, ultimately results in the inactivation of complement and produces the so-called complement-fixation reaction. Kahn[17] has also recently stated that the mechanism is one of inactivation of complement rather than of fixation by visible or invisible precipitates. He regards the Wassermann reaction as a reversible chemical reaction dependent upon the activity of the H and OH ions contributed by the various reagents, a positive reaction being due to a preponderance of H ions and a negative to OH ions. Leiboff[18] has recently reported experiments tending to show that blocking of the double bonds in the fatty acids of the lipoidal extracts employed in the Wassermann test does not materially interfere with the reaction; nor does blocking the hydroxyl groups in the lipoid molecule by substituting acetyl groups seem to interfere, from which he concludes that the Wassermann reaction must be a phenomenon of physical chemistry.

Miss Yagle has found that surface tension determinations are of no value as a means of differentiating between syphilitic and normal serum; evidently the antibody is not highly surface active or at least not more highly active than other substances found in normal non-syphilitic serum.

Of course the chemical composition of the invisible precipitate which may form in the complement-fixation reaction is unknown, but in all probability it is largely derived from the antibody serum; certainly this has been established in the case of the macroscopic serum-antiserum precipitates although these likewise contain traces of the serum antigen. The precipitates formed in the Sachs-Georgi, Meiniche and Kahn reactions in syphilis are also largely composed of serum constituents (globulins) as shown by Sachs; Epstein and Paul,[19] however, state that the precipitates are entirely of the lipoids of the antigens employed but most investigators have agreed with Taniguchi, Klosterman and Weisbach[20] and others that constituents are present from both the antibody serum and antigen.

But it is hardly profitable to discuss further the physical chemistry of the complement-fixation reaction, excellent reviews being obtainable in the references given[21]; suffice it to state that none of the

theories proposed have adequately explained the specificity of the reaction although it may be true that the *results of precipitation produce such changes in the physical chemistry of the fluids that complement is inactivated and that this constitutes what is designated as complement-fixation*. If this is true, and I believe there is some evidence in its support, complement-fixation first requires precipitation, which may or may not be visible and usually is not and of which the mechanism is unknown; this is followed in turn, not by a mechanical adsorption of complement by precipitate, but by a change in the colloidal conditions of the menstruum resulting in its inactivation. I would suggest therefore, to those trained in the methods of colloid chemistry, the advisability of studying in more detail the possible physico-chemical changes occurring in the antigen-antibody menstruum as a means for determining whether these are sufficient for causing the inactivation of complement, since the degree of precipitation is ordinarily so slight that it may not be seen even by dark-field microscopy, which greatly reduces the chances of physical adsorption of complement being the mechanism of complement-fixation.

ZONE PHENOMENA IN COMPLEMENT-FIXATION.

But before leaving this subject of physical chemistry in relation to complement-fixation it is necessary to refer to the so-called zone phenomenon (sometimes designated as "complement-deviation" or the Neisser-Wechsberg phenomenon) because it would appear to be a physico-chemical reaction sometimes encountered in complement-fixation reactions under the following conditions:

1. In titrating an extract or "antigen" for the Wassermann test to determine the "antigenic unit" it is customary to use varying amounts of the extract with a constant amount of syphilitic serum and complement. It is sometimes observed that a small amount of extract produces a stronger complement-fixation reaction than a larger amount, which is a zone reaction similar to that seen in precipitation tests employing increasing amounts of a serum antigen with a constant amount of antiserum.

Bacterial antigens however, are very much less likely to produce the phenomenon while antigens of albuminous substances like serum and blood extracts, are much more likely to produce them. In the Wassermann test the frequency of the reaction varies with different extracts and with the method employed.

2. In conducting quantitative Wassermann tests by using varying amounts of serum with a constant amount of extract "antigen" and complement, it is sometimes observed that the larger amounts of serum like 0.2 and 0.1 cc. yield weaker reactions than smaller amounts like 0.05 and 0.025 cc. This is not infrequently seen in

my quantitative method and indeed the 0.1 cc. dose may even give a falsely negative reaction. This is likewise a zone phenomenon and, I believe, constitutes an important reason for conducting all complement-fixation tests with at least two doses of serum (0.1 and 0.05 cc.) in order to reduce the error to a minimum. It is commonly believed that an excess of hemolysin due to the presence of natural antisheep hemolysin in the larger amounts of serum is responsible for the reaction but I have observed it with hemolysin-free sera and believe that it is a true zone phenomenon.

Cerebrospinal fluids, however, are much less likely to yield the reaction; in fact I have never observed it in my quantitative method employing 0.5, 0.25, 0.125 cc., etc., amounts. It is also much less likely to occur in bacterial complement-fixation reactions but occurs most frequently in tests employing albuminous antigens like serum and blood extracts.

3. When antigen, serum and complement are employed in constant amounts, the degree of complement-fixation may decrease as the amount of fluid in which the reaction takes place increases. This however, may be due to a resolution of precipitate by an excess of saline solution as seen in the titration of "antigen" for the Kahn precipitation test. It has also an important bearing upon the hemolytic activity of complement and will receive further discussion in Chapter XXI, when considering the total volume of complement-fixation tests.

4. The addition of normal serum to syphilitic serum may result in a negative Wassermann reaction when tested in amounts of 0.2 or 0.1 cc. while positive reactions occur with smaller amounts. This phenomenon described by Sellards and Minot[22] and Ronchese,[23] is also in my opinion a zone reaction due to the added colloid and as shown by Noguchi and Bronfenbrenner[34] and later by Walker, the addition of egg albumen has the same effect.

As previously stated these zone phenomena cannot be explained on the basis of the influence of an excess of hemolysin contained in the larger amounts of serum because it occurs when the serum is used in a constant dose with varying amounts of antigen or with constant amounts of antigen and varying amounts of hemolysin-free serum. Furthermore, it is hardly likely that the phenomenon is caused by the presence of antibody preventing complement-fixation or the dissociation of antigen-antibody-complement with the liberation or "unfixing" of complement; nor is it likely to be the result of an excessive absorption of antibody by the antigen rendering it impossible for complement to become attached, as suggested by Kusama.[25] In my opinion the colloids (proteins) of the serum are primarily involved although the inorganic salts may be likewise responsible and Holker's[26] explanation has impressed me as an acceptable one as follows:

"When only a minimal quantity of antigen is added to serum the euglobulin particles are precipitated, and so the amount of the precipitate is small. With increasing quantities of antigen smaller particles are included, and so the amount of the precipitate increases. With a still further increase in the quantity of antigen the electric potential on the surface of the whole of the euglobulin is reduced to zero, and therefore the precipitate reaches a maximum in quantity. With increase of antigen beyond this point, the potential on some of the serum particles is carried beyond zero and reversed by the charge on the antigen which serves to stabilize these particles. Hence the precipitate becomes smaller in amount. This process steadily goes on with increasing doses of antigen, until at a certain strength all the euglobulin particles are stabilized by adsorption of antigen, and no precipitation occurs." This explanation has been offered in relation to the zone phenomenon in serum precipitation reactions and I believe it is likewise applicable to complement-fixation since both reactions are so intimately related.

AUTHOR'S THEORY OF THE MECHANISM OF SPECIFIC COMPLEMENT-FIXATION.

My interpretation of the data and opinions expressed in this and preceding chapters has lead to the following theory of the mechanism of specific complement-fixation reactions, which includes all except the Wassermann reaction and those exhibited by the *normal* sera of some of the lower animals:

When antigen and antibody serum are brought together in suitable proportions a specific reaction occurs which may or may not be followed by microscopic precipitation but results in a change in the chemistry of the menstruum capable of *inactivating the complement enzyme*. A fixation of complement by mechanical adsorption by precipitate is not an essential part of the mechanism but may occur as a secondary phenomenon. The essential change is one involving the chemistry of the menstruum resulting in the inactivation of the complement enzyme. When corpuscles and hemolysin (amboceptor) are added after the first phase or primary incubation during which these changes occur, the complement enzyme is unable to hemolyze the corpuscles even though they have been "sensitized" or prepared by a neutralization of their anti-complement enzyme by the specific hemolysin as explained on page 38. Therefore hemolysis does not occur or is incomplete and a "positive reaction" results. If the serum contains no antibody or an amount too small to bring about the chemical change in the menstruum and if the antigen alone and serum alone are unable to inactivate the complement, complete hemolysis or a "negative reaction" is the result.

AUTHOR'S THEORY OF THE MECHANISM OF THE WASSERMANN REACTION.

The mechanism of complement-fixation in syphilis is essentially similar. If the antigen is a saline or watery extract of *Spirochæta pallida* a specific reaction may occur as described above between the antibody and an antigen of the proteins or a protein-lipoid complex of the organisms. But these reactions are weak, irregular and of no practical value so that the Wassermann reaction ordinarily means an interaction between antibody and the non-specific lipoids derived from either syphilitic or non-syphilitic tissues, the latter usually yielding the better "antigen." Certainly alcoholic extraction of syphilitic liver or other tissue does not appear to produce an antigen in any sense "specific" or superior to similar extracts of non-syphilitic tissue in the same state of autolysis.

A characteristic quality of the antibody of *Spirochæta pallida* and *Spirochæta pertenuis* (yaws) is its property of reacting with the lecithins (phosphatids) and other lipoidal substances in watery or alcoholic extracts of either syphilitic or non-syphilitic tissues. When such extracts and syphilitic serum are brought together in proper proportions a reaction occurs which may or may not be followed by microscopic precipitation but *resulting in a change in the chemistry of the menstruum capable of inactivating the complement enzyme.* This prevents the hemolysis of corpuscles sensitized or prepared by specific hemolysin or amboceptor and results in a *positive* Wassermann reaction. If the serum contains no antibody or an amount too small to produce the changes in the menstruum capable of inactivating the complement enzyme and if the antigen alone and serum alone are unable to inactivate the complement (that is, are free of anticomplementary effects), the corpuscles added after the primary incubation undergo complete hemolysis (*negative* Wassermann reaction) when they have been adequately prepared or "sensitized" by adding specific hemolysin (amboceptor) which neutralizes their anticomplement enzyme. With the assistance of Mr. Herman Brown, I have observed however, that the fixation of guinea-pig complement by syphilis antibody and Wassermann antigen does not affect the lipase and protease in either human or guinea-pig serum; in other words if complement is an enzyme inactivated by the process of complement-fixation the changes do not appear to influence these well known serum enzymes. Furthermore while complement in guinea-pig serum is inactivated by extraction with chloroform to remove antiferment, the serum protease is not affected thereby. Wormall, Whitehead and Gordon[27] have recently reported that the ratio of proteoclastic and anticomplementary powers of pancreatic extracts are almost identical.

Since the lipoids are not immunologically specific and since the

reaction occurs with the lipoids of healthy non-syphilitic tissues, the complement-fixation reaction in syphilis is biologically non-specific, just as the Sachs-Georgi, Meinicke, Kahn and other precipitation reactions in this disease are biologically non-specific.

The antibody is produced with certainty only in syphilis and frambesia tropica (yaws) which accounts for its very high degree of practical specificity in the serum diagnosis of syphilis. But the source of this antibody is obscure and constitutes an essential part of this theory upon the mechanism of the Wassermann reaction.

I believe that there is a close analogy between the production of the Wassermann antibody in human beings, rabbits and monkeys by *Spirochæta pallida* and the production of heterophil antibody in rabbits by extracts of the tissues of the guinea-pig, horse, dog, cat, bacteria, etc. In other words a lipoid-protein complex of the spirochetes of syphilis and yaws may act as a heterophil or Forssmann antigen capable of causing the production of a heterophil antibody (the Wassermann antibody) in man and to a lesser extent in rabbits and monkeys. The chief quality of this antibody is its ability to react with lipoids and especially lecithins in the precipitation and complement-fixation reactions of syphilis.

An objection to this theory is that the antibody reacts not only with the lipoid complexes of *Spirochæta pallida* and syphilitic tissues but likewise with lipoids from the normal tissues of many different animals. In other words it is non-specific. But the same is true of the heterophil hemolysins since the injection of rabbits with extracts of liver and kidney of the guinea-pig results in the production of antichicken as well as of antisheep hemolysin. Furthermore, as discussed in Chapter IV, the injection of rabbits with extracts of guinea-pig tissues may produce the Wassermann antibody as claimed by Taniguchi and indicating that three different heterophil antibodies may be produced in the rabbit by injections of a single heterophil antigen composed of an extract of guinea-pig tissue.

MECHANISM OF COMPLEMENT-FIXATION WITH NORMAL HUMAN SERA; PROTEOTROPIC REACTIONS.

But if the Wassermann antibody is heterophil in nature the question naturally and immediately arises whether other organisms or foreign substances aside from *Spirochæta pallida* and *Spirochæta pertenuis* are capable of producing it in man and thereby yield positive reactions in diseases other than syphilis and yaws. That fully 90 per cent of human beings are subject to stimulation by some kind of heterophil antigen is suggested by the presence of natural antisheep, antiguinea-pig and other natural hemolysins in their sera. This question can be answered only on the basis of experience with the Wassermann test and while falsely positive

reactions have been reported as occurring in leprosy, tuberculosis, scarlet fever, tumors, trypanosomiasis and almost all other diseases of mankind, yet I believe it may be stated that the majority of such reports have been due to technical errors in conducting the tests and that only in leprosy and trypanosomiasis *may* one detect in the sera an antibody capable of sometimes yielding a true complement-fixation reaction with the Wassermann "antigens." But even in these diseases (at least in leprosy) it is possible to avoid the reactions by technical details unless the leper is also syphilitic, in which case a true positive Wassermann reaction occurs.

Of course this subject intimately concerns the specificity of the Wassermann reaction which is discussed with more detail in Chapter XL because of its great practical importance, but here it may be stated that outside of leprosy and trypanosomiasis we have no proof that any organisms other than the spirochetes of syphilis and yaws are capable of producing the Wassermann antibody. Certainly there is no such thing as natural syphilis antibody in man and a truly positive Wassermann reaction is to be regarded as an indication of the presence of syphilis when yaws and leprosy can be excluded.

The complement-fixation reaction in the bacterial diseases is even more specific but unexpected positive reactions may occur if the serum being tested contains a natural antibody. For example many human sera contain natural typhoid agglutinins but in my experience these have not yielded positive complement-fixation reactions unless the titer was 1 to 30 or higher. But among cattle positive tuberculosis complement-fixation reactions have occurred when no lesions were discoverable at necropsy and these may have been due to natural antibody (see Chapter XLVI). Guinea-pig sera do not contain natural syphilis antibody but the possibility of their containing natural antibody for various bacterial antigens is of some importance in relation to their use as complement, which is discussed in Chapter XI.

This does not mean, however, that the sera of apparently healthy human beings may not give unexpected positive Wassermann reactions. Indeed this happens almost weekly in laboratories conducting large numbers of tests but with technically correct methods their occurrence is usually due to the presence of concealed or so-called "latent" syphilis which may not have been clinically suspected.

Aside from these truly positive reactions however, weakly positive reactions may sometimes occur with the sera of non-syphilitic individuals, but their frequency is more or less in relation to the technique employed and the skill of the serologist. But even when both of these are acceptable, reactions of this kind may occur when the sera are so slightly anticomplementary as to give weakly positive reactions in the presence of antigen although the serum controls

show tardy but finally complete hemolysis; also, and more frequently in my experience, when the complement is defective, that is, poor in hemolytic activity and unduly sensitive to inactivation by antigen alone. These reactions however, do not involve the question of antibody but rather indicate that physico-chemical changes may occur in serum capable of inactivating complement, especially a weak complement enzyme, and thereby yield a reaction resembling a positive Wassermann reaction.

But there is a type of non-specific reaction sometimes exhibited by the sera of normal human beings which demands special attention, namely, that sometimes shown by raw or unheated serum tested with alcoholic tissue extracts ("antigens") employed in the Wassermann test. Sachs and Altmann[28] and Boas[29] first drew attention to them and Noguchi thought that they were in some way related to the presence of protein in the antigen, for which reason they were designated as "proteotropic reactions."

In my experience they are more likely to occur with crude alcoholic extracts and especially those reënforced with cholesterol, than with the acetone-insoluble lipoids which always contain much less protein. As shown by Browning, Dunlop and Kennaway[30] however, the *concentration of alcohol* is a determining factor and indeed Boas has stated that alcohol alone in saline without lipoids may yield the reaction. It is possible therefore, since plain alcoholic extracts are ordinarily used in 1 to 5 to 1 to 10 dilutions, that the alcohol is a more important factor than the proteins. In my experiments when absolute ethyl alcohol was titrated for anticomplementary effects in amounts varying from 0.5 cc. of 1 to 2 to 1 to 256 (diluted with saline in the same manner as antigen), the unit was 0.5 cc. of 1 to 4. When used in dose of 0.5 cc. of 1 to 12 (one-third anticomplementary unit) with the unheated sera of healthy non-syphilitic individuals weakly positive reactions occurred in approximately 5 per cent, corroborating the observations of Boas. The positive reactions usually occurred only in the higher dilutions of each serum, suggesting zone reactions; with heated serum only negative reactions were observed. With the sera of Wassermann-positive individuals the incidence of positive reactions with antigen of pure alcohol was slightly higher and they occurred with the larger amounts of serum like 0.1 cc. and 0.05 cc.; with heated syphilitic sera the reactions were negative.

Heating serum at 55° C. for as short a period as five to ten minutes will prevent the occurrence of these reactions with alcohol or tissue extracts as shown by Rule, Trist and myself;[31] this is in sharp contrast to the influence of heat upon the sera of rabbits, dogs and mules which tends to increase rather than decrease their capacity for producing non-specific positive reactions.

While the exact mechanism of these reactions is unknown yet they unquestionably bear a direct relation to the kind of antigen employed; Noguchi's extract of acetone-insoluble lipoids produces them but rarely as likewise my new cholesterolized and lecithinized alcoholic extract of beef heart. It may be that the proteins of plain or crude extracts are responsible as Noguchi suggests, but if so, the mechanism is obscure. One naturally inquires if a serum yielding a reaction of this kind contains precipitin for beef protein since antigens are usually prepared of beef heart; in my experience precipitation could not be demonstrated and furthermore, the reactions have occurred with alcoholic extracts of human heart muscle (homologous protein). It would appear that the reaction is due to an obscure physico-chemical state of raw serum involving the proteins, influenced by the alcoholic content of the antigen and removable by heating at 55° C. for ten minutes, the latter constituting one of the most important reasons for heating sera for the Wassermann test.

AUTHOR'S THEORY OF THE MECHANISM OF COMPLEMENT-FIXATION WITH NORMAL ANIMAL SERA.

But any discussion of the mechanism of complement-fixation would be incomplete if ignoring those reactions sometimes yielded by the *normal* sera of some of the lower animals. These are indeed the most puzzling of all because they occur with the sera of apparently healthy animals and have no relation to their diseases; furthermore, as discussed in Chapter XXVIII, they possess a high degree of practical importance in relation to the use of the sera of rabbits and other of the lower animals for complement-fixation tests after immunization or the experimental production of disease (syphilis, yaws, trypanosomiasis, etc.), as well as in diagnostic complement-fixation tests for certain diseases occurring in mules, cattle, etc.

In my experience these reactions are more likely to occur with antigens of staphylococci, typhoid bacilli and other bacteria than with the alcoholic tissue extracts employed in the Wassermann test; of the latter those containing 0.4 per cent cholesterol react more frequently than plain alcoholic extracts and those containing 0.1 to 0.2 per cent cholesterol.

The sera of rabbits are especially likely to yield the reaction. I have found the sera of dogs and mules ranking next in frequency but those of the lower apes, goats, sheep and chickens may sometimes yield positive reactions. In my experience the sera of horses are not likely to yield the reaction; swine sera do so only occasionally and those of guinea-pigs almost never, which constitutes an import-

ant reason for using them as complement in the Wassermann and other complement-fixation tests.

Fresh unheated sera are much less likely to yield positive reactions than heated sera; some observers have stated that they do not react at all but I know this is untrue insofar as the sera of normal rabbits, dogs and mules are concerned. Heating at 55° C. for fifteen to thirty minutes results in the strongest reactions; heating at 60° C. for thirty minutes reduces their frequency and strength while heating at 62° C. for thirty minutes usually abolishes the reactions especially in the case of rabbit, cattle and mule sera.

The mechanism of these reactions is obscure. Miss Yagle and I have observed however, that the sera of normal rabbits yielding positive Wassermann reactions have usually yielded positive Kahn precipitation reactions; furthermore, tests conducted with these sera after precipitation have usually yielded negative reactions (some were anticomplementary), suggesting that an antibody was present identical in both the precipitation and Wassermann reactions and removable by absorption with the same extract. We have also observed that the absorption of rabbit sera with staphylococci has sometimes weakened or abolished the complement-fixation reaction with staphylococcus antigen. Miss Richter, Miss Rule and I have also found that as reported by Sachs, treating normal rabbit sera with 8.2 volumes of $\frac{N}{300}$ hydrochloric acid in distilled water, allowing to stand one-half hour, removing the precipitates and employing the supernatant fluids to which is added 0.8 volumes of 10 per cent sodium chloride solution, sometimes removes a portion of the substance responsible for the reaction (see Chapter XXXVIII). Evidently this method results in a precipitation and removal of some of the protein constituents responsible for the reaction or in a physical removal of antibodies in the precipitates (presumably largely composed of the globulins of the serum).

These results suggest that the reactions may be due to the presence of natural complement-fixing antibodies which in the case of the Wassermann reaction must be heterophil in nature, since rabbits do not suffer spontaneously with syphilis and it is known that *Spirochæta cuniculi* sometimes producing a venereal disease among them bears no relation to the reactions. But the identity of the heterophil antigen is unknown as likewise the mechanism of the influence of heat upon the serum; the latter however, may be due, at least in part, to the inactivation of natural complement because it is well known that if fresh normal rabbit, dog or mule sera are tested unheated or in the presence of an excess of complement these non-specific complement-fixation reactions are not likely to occur and may be entirely avoided.

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CHAPTER VII.

NATURE AND MECHANISM OF ANTICOMPLEMENTARY ACTION.

In every complement-fixation test at least two controls should be included. One of these is designated as the "serum control" set up with the dose of serum or spinal fluid and complement but with no antigen; the second is the "antigen control" set up with the dose of antigen and complement but with no serum. Both are given the same primary incubation as the main test, receive the usual amounts of hemolysin and erythrocytes, followed by the secondary incubation and a reading of the reactions. *Both should show complete hemolysis* and thereby prove that the serum or spinal fluid *alone* and the antigen *alone*, in the amounts employed, are without effect upon hemolysis, that is, are free of "anticomplementary" activity.

In other words under these conditions the serum or spinal fluid being tested is of *itself* unable to inactivate the complement in the amounts employed. But sometimes a control shows an inhibition of hemolysis indicating that the serum or spinal fluid contains something interfering with the phenomenon and constituting "anticomplementary action." Obviously under these conditions the main tests with a serum or spinal fluid of this kind will likewise show an equal or slightly greater degree of inhibition of hemolysis which, without the control, would lead to the error of recording a falsely positive reaction.

Complete hemolysis in the antigen control tube indicates that this reagent in the amount employed is alone without an inactivating effect upon the complement; if it were otherwise, all of the main tests would likely show an equal degree of interference of hemolysis resembling positive complement-fixation reactions.

Not only may a serum, spinal fluid or antigen become anticomplementary but even a saline solution may be found to have the same effect. Furthermore, the complement of some guinea-pigs is sometimes unduly sensitive to the anticomplementary action of these different reagents and the phenomenon obviously has a place of great practical importance in serum diagnosis by complement-fixation.

Definitions.—By anticomplementary action is meant therefore, that a serum, spinal fluid or other fluid being tested, as well as the antigen

being employed, may contain substances or undergo some peculiar change whereby they are *alone* capable of interfering with serum hemolysis by inactivating the complement. The reaction is called "anticomplementary" because it can be shown that the results are due to an effect upon the complement rather than upon hemolysin or corpuscles.

But this phenomenon should not be confused with the *antihemolytic* action of serum and other albuminous solutions. For example, a perfectly fresh serum free of anticomplementary effects may still protect erythrocytes against many different hemolytic agents. As stated in Chapter II a large number of substances from the animal, vegetable and mineral kingdoms are capable of hemolyzing washed erythrocytes in the test-tube. In relation to the complement-fixation reaction one may mention alcoholic and saline extracts of various tissues employed as "antigens" in the Wassermann test, toxins and other substances in bacterial antigens, acids or alkalies in glassware, etc. But if serum is added to the corpuscles or if unwashed cells are employed, hemolysis is reduced or prevented. In other words, serum is *antihemolytic* probably because its proteins or protein-lipoid complexes form new compounds with the hemolytic agents, sometimes seen as precipitates, which are much less destructive for erythrocytes. But an anticomplementary serum is not usually any more antihemolytic than one that is free, anticomplementary action indicating that the phenomena are probably not due to the same substances and that the terms therefore are not synonymous as sometimes stated.

The phrase "anticomplementary substances" is however, somewhat cumbersome and the word *antilysins* has come into general use as a substitute. It is acceptable providing one bears in mind that the substances are antilytic because they are anticomplementary.

Influence of Technique upon Anticomplementary Action.—It is readily understood that the technique of complement-fixation tests exerts a marked effect upon anticomplementary reactions. This is especially true in relation to the amounts of complement and hemolysin employed. A serum or antigen may contain but small amounts of anticomplementary substances but if relatively large amounts of complement or hemolysin or both are used, the effects may not be manifested in the test-tube. Furthermore, the kind of primary incubation has an influence and likewise the amounts of serum or antigen employed, etc. It is not surprising therefore, that one complement-fixation method may give a higher percentage of anticomplementary reactions than some other method or that a given serum is found anticomplementary in one laboratory and not in another. Furthermore, the sera of guinea-pigs employed as complement may vary in susceptibility to the anticomplementary

substances of serum and antigen and it is not unusual to observe some sera or antigens anticomplementary one day but not the next day when retested with another complement serum. By using a mixture of sera from healthy full-grown unused pigs the influence of this factor may be reduced to a minimum and the technique of complement-fixation employed should be one not too sensitive to the antilysins of serum, spinal fluid, antigen and saline solution.

Influence of Antigen Upon Anticomplementary Reactions.—But it is especially important to remember in connection with this subject of the influence of technique that a serum or spinal fluid may not show any more evidence of anticomplementary action in the serum control tube than a slower rate of hemolysis which, however, is completed in the usual hour of incubation, while those tubes carrying the serum and antigen show a slight degree of inhibition of hemolysis that may lead the inexperienced to report a weakly positive complement-fixation reaction. This kind of reaction is probably due to the summation of the anticomplementary activity of serum and antigen even though each alone results in complete hemolysis in their respective controls.

THE ANTICOMPLEMENTARY ACTIVITY OF SERUM.

Since complement is an extremely sensitive reagent it is not surprising that the majority of sera can be shown to possess some degree of anticomplementary action if tested in large amounts.

Fresh Serum.—But the perfectly fresh sera of human beings are generally free of anticomplementary activity in the amounts commonly employed in the Wassermann and other complement-fixation tests. Occasionally however, exceptions to this rule are encountered and fresh sterile sera are found to be anticomplementary in an unheated state, as well as after being heated at 55° C. for fifteen to thirty minutes and indeed even after heating at 62° C. for thirty minutes. It is impossible to express the incidence because the technique of complement-fixation greatly influences the results; in my method it is encountered about once in every 2000 to 3000 tests. Sanford[1] has reported a number of such individuals and I have had several cases of the same kind during the past three years. All of the latter were known to have syphilis and were receiving intravenous injections of neoarsphenamine. The majority of Sanford's cases were likewise syphilitic and it may be that the anticomplementary state of their freshly drawn serum bore a relation to this disease or in some instances to the administration of the drugs employed in treatment.

It is sometimes stated that if blood is drawn after a meal that the *chylous serum* is apt to be anticomplementary but this has not been

my experience. Curiously enough, however, serum separated from its clot and tested within an hour or two after bleeding is more apt to be anticomplementary than when the clot has been left over night or a few days in a refrigerator.

Apparently the changes in serum responsible for the phenomenon are not constant since the serum may be anticomplementary at one time and not at another. Of course these variations may be due to the complement employed in tests on different days but I have had three cases in which every test over months of time showed an unvarying and persistent anticomplementary state of the serum.

The fresh sterile sera of rabbits and dogs are more apt to prove anticomplementary in my experience than human sera.

Preserved Sterile Serum.—All sterile sera may acquire anticomplementary properties, the rapidity and degree depending upon the temperature at which they are kept. Thus at 38° C. they may become anticomplementary within three days and at room temperature (20° to 22° C.) in about five to seven days; at low temperatures like 0° to 8° C. the changes may not occur over a period of several weeks or months. But the conditions governing these changes are not at all understood and the situation is full of surprises. Not infrequently a serum is found to acquire anticomplementary properties under the best of conditions while another, supposed to be surely anticomplementary, proves otherwise in the actual test.

In my experience *sera left on their clots are much less likely to become anticomplementary than separated sera.* This is an important practical point in relation to the shipping of specimens of blood to distant points for the Wassermann test since whole blood is apt to be more satisfactory than the separated serum.

But while sera containing traces of hemolyzed corpuscles are not usually anticomplementary, those containing *large amounts of hemoglobin* and other disintegration products of erythrocytes, are apt to be strongly anticomplementary both before and after heating at 55° C. For this reason serum for the complement-fixation test should be separated from its clot before marked hemolysis has occurred.

As a general rule the anticomplementary activity of preserved *sterile* serum is removed by heating at 55° C. for fifteen to thirty minutes; therefore, the substances responsible are designated as *thermolabile.* Heated serum is just as likely subsequently to acquire anticomplementary properties as unheated serum.

The addition of a *preservative* like phenol, cresol, tricresol, chloroform, etc., to serum does not materially retard or prevent the acquisition of thermolabile anticomplementary activities. Indeed the addition of these in concentration of 0.5 per cent or more may increase the action probably because of a destruction of complement by the chemical agent, but concentrations varying from 0.1 to 0.3

per cent are not anticomplementary and usually suffice for antiseptic or bacteriostatic purposes.

Dried Serum.—Dried serum does not acquire anticomplementary properties although during the process of drying such may occur unless desiccation is conducted at a low temperature and preferably under vacuum to hasten the process.

Contaminated Serum.—The mere presence of a few bacteria like staphylococci in serum may not render it anticomplementary as long as their proliferation is prevented. Therefore, serum for the Wassermann and other complement-fixation tests is generally satisfactory if tested within a few days after collection of blood even though bacterial contamination has occurred. But after the natural bacteriostatic properties of serum have become exhausted and bacteria are permitted to multiply rapidly, anticomplementary properties develop especially in the presence of staphylococci. Furthermore, the anticomplementary state is *thermostabile*, that is, is not usually removed, although somewhat reduced, by heating the serum at 55° C. for thirty minutes. As shown by Wilde[2] and later by Craig,[3] Kyutoku,[4] Uchida[5] and others, many different bacteria are capable of producing these effects and it would appear that the bacterial bodies are more important in this connection than the soluble toxins produced by them in the serum.

Influences of Disease Upon the Anticomplementary Properties of Serum.—While it may be that syphilis bears a relation to those occasional instances of anticomplementary state of perfectly fresh and sterile sera previously mentioned, yet the relationship of other diseases to this quality of serum is not clear and in my experience pathological conditions do not appear to have any definite bearing upon the phenomenon. Becker[6] however, has recently reported a series of 112 anticomplementary reactions with the sera of 69 individuals, 48 per cent of whom were syphilitic and the majority of the balance of whom presented evidence of various chronic bacterial infections.

In the acute infections like typhoid fever, scarlet fever, diphtheria, pneumonia, malaria, etc., the serum has been uniformly free of enhanced anticomplementary properties in my experience even when collected at the height of fever. In leprosy and tuberculosis however, I believe that the anticomplementary property may be sometimes slightly increased and especially in the presence of cholesterolized antigen, which probably accounts in part for the increased tendency for falsely positive Wassermann reactions in these diseases.

But in diabetes mellitus and nephritis the serum is not usually affected even when the blood contains large amounts of glucose, urea, etc. Uchida has observed that the addition of large amounts of glucose, urea, creatin and creatinin to serum did not increase

anticomplementary properties in my test. The addition of uric acid and cholesterol however, had an appreciable influence. Sometimes serum deeply tinged with biliary products during severe jaundice is of increased anticomplementary action but this is the exception rather than the rule in tests employing fresh serum. The presence of malignant tumors does not appear to have any effect upon the phenomenon.

Influence of Pregnancy. –It is possible that changes in the serum during pregnancy and especially the latter months may account for the numerous reports to be found in literature regarding falsely positive Wassermann reactions in this condition. But this tendency is certainly not in evidence in my new test and the serum does not appear to have any enhanced anticomplementary properties even when blood is taken during the early and late toxemias of pregnancy.

However, the *sera of placental and cord bloods* collected at the time of delivery are sometimes anticomplementary and must be carefully watched when submitted to the complement-fixation test.

Postmortem Sera.—The sera from specimens of blood collected after death are sometimes anticomplementary and especially those which are hemolyzed or putrid. Specimens collected within a few hours after death and usually from the heart are generally free of anticomplementary activity but on the whole postmortem specimens must be carefully watched.

Influence of Absorbing Natural Hemolysin Upon the Anticomplementary Action of Serum.—The absorption of natural antisheep or other hemolysins by adding the corresponding corpuscles sometimes renders a serum anticomplementary, the so-called "Sachs-Friedberger phenomenon." In practically all instances however, this induced anticomplementary condition is removed by heating at 55° C. for ten to fifteen minutes, that is, the antilysin or antilytic state is thermolabile. For this reason I always remove natural antisheep hemolysin from the raw serum according to the method described in Chapter XXXI and heat afterward.

The mechanism of this phenomenon is not understood. Miss Yagle and I have observed that absorbing serum with *unwashed* sheep corpuscles is always more likely to produce it than absorption with cells washed at least three times and suggesting that the reaction may be due to carrying over into the human serum traces of anticomplementary sheep serum clinging to the corpuscles; under these circumstances the induced anticomplementary state of the serum is sometimes thermostabile. Corpuscles washed more than three times to remove all traces of serum proteins in the wash fluids, but rarely render sera anticomplementary. One naturally surmises that the phenomenon may be due to the formation of precipitates between the sheep serum adhering to the corpuscles and the human serum but Miss Yagle and I have not been able to detect any evidences of precipitation in our experiments.

THE ANTICOMPLEMENTARY ACTIVITY OF SPINAL FLUID.

As previously stated freshly drawn cerebrospinal fluid is rarely anticomplementary; I have never encountered a single one but Sanford has reported several occurring in syphilis and believes that the anticomplementary state may bear a relation to this disease.

In my experience, *clear* spinal fluids kept at a temperature sufficiently low to prevent bacterial growth do not acquire anticomplementary properties for long periods of time and not nearly as frequently or rapidly as sera kept under the same conditions, even though they may contain considerable protein (as in paresis) and viable bacteria (like the skin cocci) as detected by culture. Therefore spinal fluids are not routinely heated at 55° C. preliminary to the Wassermann test as is the usual custom with sera.

But spinal fluids from cases of septic meningitis, cloudy or purulent with pus and bacteria, are much more likely to acquire anticomplementary properties than the fluids of normal or syphilitic individuals. Likewise when the latter become cloudy by the proliferation of contaminating bacteria, thermostabile anticomplementary substances are usually found to be present.

Fresh and unheated spinal fluids of normal rabbits and dogs are not anticomplementary in amounts of 0.5 cc. or less, but after heating at 55° C. for thirty minutes this property is sometimes acquired by them but not nearly as frequently or as intensely as in the case of their sera.

THE ANTICOMPLEMENTARY ACTIVITY OF MILK, URINE, TRANSUDATES AND EXUDATES.

Milk, sometimes employed in complement-fixation tests in the course of investigations and especially in syphilis, is not highly anticomplementary in the *fresh* state. But after the bacteria have had an opportunity for multiplying it becomes more and more anticomplementary even after being heated at 55° C. for thirty minutes. For this reason *it is usually advisable to determine the anticomplementary unit of each specimen preliminary to the main tests* and to employ one-half to one-quarter these amounts; at least Klauder and myself[7] were unable to conduct the tests with any single fixed amount because of the very considerable variation in the anticomplementary properties of different specimens.

Urine is even more likely to vary in its anticomplementary properties and demands a preliminary titration of each specimen for the anticomplementary unit. Perfectly fresh urine is of course much less anticomplementary than older specimens in which bacterial proliferation and the deposition of salts with marked changes of pH have occurred.

Transudates like peritoneal and edema fluids (ascites) are, like fresh blood sera, usually free of anticomplementary properties in the amounts commonly employed in complement-fixation tests. The same is true of aqueous and vitreous humors of the eye but *saliva* and *semen* are very highly anticomplementary even in a fresh state, probably because of the presence of bacteria.

Exudates vary greatly in anticomplementary properties, as would be expected, probably because of their bacterial content. Ogawa[8] observed that the anticomplementary units of tuberculous pleural exudates varied from 0.06 to 1 cc. when tested according to the technique of my new method. *Chancre juices* also vary greatly in anticomplementary properties as observed by Klauder and myself.[9] For this reason it is almost impossible to settle upon a fixed dose for testing fluids of these kinds and it is necessary to either determine their anticomplementary units before the main tests or to employ them in varying amounts with a control on each dose as described in Chapter XXXVIII.

THE ANTICOMPLEMENTARY ACTIVITY OF ANTIGENS.

Substances commonly employed as antigens in complement-fixation tests are likewise usually anticomplementary in some amount. This is especially true of the various alcoholic and other extracts of syphilitic and non-syphilitic tissues employed in the Wassermann test; also of the various bacterial antigens.

For this reason it is essential to determine the smallest anticomplementary amount of antigen employed in a complement-fixation test, designated as the "anticomplementary unit," and to very carefully avoid using the antigen in this amount in conducting the main tests. Furthermore, a control on the antigen alone should be included in all tests in order to prove that it is free of anticomplementary effect in the amount employed rather than to assume that this is the case.

The method of preparing antigens has considerable influence upon their anticomplementary effects and every effort should be made to reduce their anticomplementary action to a minimum while increasing their antigenic sensitiveness in order to give the best possible ratio or "antigenic index" between the two. For example, if an antigen is anticomplementary in a dose of 0.5 cc. of 1 to 5 dilution and antigenic in a dose of 0.5 cc. of 1 to 3000 the "antigenic index" is 600, meaning that the antigenic unit is six hundred times less than the anticomplementary unit. This large leeway between the two is always advisable—the greater the better—because it permits one to use the antigen in a dose of 10 or more antigenic units without running the risk of producing non-specific or falsely positive complement-fixation reactions due to the anti-

complementary effects of antigen. For instance a second antigen may be anticomplementary in a dose of 0.5 cc. of 1 to 20 and antigenic in a dose of 0.5 cc. of 1 to 1000 giving an antigenic index of but 50 and thereby much inferior to the first mentioned.

THE ANTICOMPLEMENTARY ACTIVITY OF SALINE SOLUTION.

Because complement is so easily inactivated it is not surprising that the saline solution employed in complement-fixation tests may be sometimes responsible for anticomplementary effects, usually suspected whenever the complement and hemolysin give unusually low titers.

Mason and Sanford[10] have thought that the effects of such saline solutions upon complement may be due to their pH concentration but, as I shall point out in Chapter VIII, this is hardly the case under routine conditions. My experience agrees rather with that of Kellogg and Wells[11] who showed that the chemical constitution of the water with particular reference to its content of magnesium was the determining factor. They found that saline solution carrying from 0.05 to 0.38 gm. of either magnesium chloride or sulphate per 1000 cc. gave the best complement and hemolysin titers; less than 0.02 gm. per liter has no effect while more than 0.38 gm. had the opposite effect and rendered the solution antilytic. This probably accounts for the fact that when saline solution is prepared of raw water it is sometimes superior to that prepared of distilled water; at least I have known this change in technique to solve the difficulty in several laboratories and if saline solutions prepared with distilled water and chemically pure sodium chloride give unusually low complement and hemolysin titers it would appear advisable to add 0.1 gm. of either magnesium sulphate or chloride to each 1000 cc. as advised by Kellogg and Wells. Otherwise merely to use a natural raw water in preparing physiological saline solution instead of distilled water may solve the difficulty.

PROPERTIES OF ANTICOMPLEMENTARY SUBSTANCES.

Naturally many efforts have been made to devise ways and means for removing anticomplementary substances from sera and antigens without removing complement-fixing antibodies from the former at the same time, and most of our information upon the properties of the serum antilysins has been gained in this manner.

Influence of Heat.—Heating *human* serum at 55° C. for ten to fifteen minutes removes the *thermolabile* antilysins and is usually sufficient for routine purposes. The *thermostabile* antilysins like those in contaminated sera are much more resistant, are usually unaffected by heating at this temperature and may require exposure

to temperatures as high as 90° C. for thirty minutes for removal. Unfortunately however, this temperature also destroys antibody and the method therefore is without practical value.

Heating *human* sera never has the reverse effect of rendering them anticomplementary but the exposure of the sera of *rabbits, dogs, sheep* and *cattle* to 55° C. sometimes has this effect and especially in the case of rabbit sera heated at 55° C. for thirty minutes. Heating at 60° to 62° C. for thirty minutes reduces the anticomplementary activity of these animal sera to some extent but higher temperature are required for abolishing them altogether. Furthermore while the unheated spinal fluids of normal dogs and rabbits are almost always free of anticomplementary effects, yet after heating at 55° C. for fifteen to thirty minutes they are usually found to have become anticomplementary and especially when 0.5 to 1 cc. amounts are employed.

Why heating sometimes unmasks the antilysins of animal sera is unknown. Doubtless in some instances the absence of anticomplementary action of unheated human or animal serum is due to the presence and influence of its complement, a natural hemolysin or both upon the hemolytic system being employed but the unmasking of antilysins in rabbit and other animal sera by heat is not so easily explained and probably involves changes in the colloids as the basic mechanism.

Heating antigens for the Wassermann and other complement-fixation tests may slightly reduce their anticomplementary effects but complete removal, even by boiling them, is not usually observed.

Influence of Absorption.—Adding such substances as barium sulphate, animal charcoal or kaolin to anticomplementary sera may sometimes remove a portion of the antilysins; but complement-fixing antibody may be absorbed at the same time and the method therefore is without practical value. Indeed the absorption of fresh serum with such substances may render it anticomplementary although this induced state is usually thermolabile.

Noguchi[12] has observed that the absorption of animal sera with washed sheep corpuscles may remove the antilysins and that the corpuscles were thereby rendered more resistant to hemolysis. Zinsser and Johnson[13] were unable to confirm these results insofar as the thermolabile antilysins of human sera were concerned and Miss Yagle and I have failed to confirm them not only in relation to the thermolabile and thermostabile antilysins of human sera, but likewise in the case of the antilysins of heated dog, ox and rabbit sera as well. Indeed the absorption of unheated human and animal sera with washed sheep corpuscles may render them anticomplementary (thermolabile) as previously described and no method has been developed for removing the serum antilysins by absorption or adsorption processes.

Influence of Filtration.—The removal of precipitates from sera by centrifuging or paper filtration usually reduces their anticomplementary effects. Kyotoku also observed in my laboratory that filtration through small, sterile earthen candles tended to remove thermostabile antilysins and sometimes without removal of antibody at the same time but in numerous experiments I have found that while filtration of diluted sera through these candles, as well as through Mandler and Berkefeld filters, usually removed the antilysins, that complement-fixing antibodies were held back at the same time. Uchida[14] has also observed in his experiments upon the isolation of the typhoid complement-fixing antibody that filtration of saccharose extracts of sensitized bacilli through Mandler filters removed not only anticomplementary substances from the solutions but dissociated agglutinin and complement-fixing antibody as well.

Influence Upon the Complement-fixation Antibody.—Of considerable practical importance in relation to special methods adapted for conducting complement-fixation tests with anticomplementary sera and spinal fluids, is the question whether or not the antilysins have an injurious effect upon antibody. After sera have stood for a few days to a week or so in a refrigerator it is not uncommon to observe them yielding stronger complement-fixation reactions than in a perfectly fresh state but I believe[15] this is due to the acquisition of an anticomplementary action too slight for showing inhibition of hemolysis in the serum control tube, but yet sufficient for adding to the degree of inactivation or fixation of complement in the presence of antigen and thereby yielding stronger reactions. Uchida[16] has observed in my laboratory that anticomplementary substances developed in sera and spinal fluids, including those resulting from bacterial contamination, have no direct destructive effect upon the antibody concerned in the Wassermann test although the latter gradually deteriorates in preserved serum and spinal fluid as is true of antibodies in general under similar conditions.

THE CHEMISTRY OF ANTICOMPLEMENTARY SUBSTANCES.

Since the antilysins of serum and spinal fluid have never been isolated it is impossible to state their chemical nature in exact terms. Noguchi has thought that the thermostabile antilysins of dog and other animal sera were associated with the lipoidal constituents of serum (cholesterol, fatty acids, neutral fats and less defined bodies), because extraction with ether, benzol and acetone appeared to remove them, but Zinsser and Johnson have found the thermolabile antilysins of human serum associated with the globulin fractions and this has certainly been the general result of my experiments with both thermolabile and thermostabile antilysins of human serum and spinal fluid. Kyotoku, employing the refractometric

method of Robertson in a study of the proteins of anticomplementary human sera, also came to this general conclusion and furthermore observed that the state bore no constant relation to changes in hydrogen-ion concentration, although anticomplementary sera gradually become more alkaline.

Several years ago Sachs[17] described a useful method for removing thermostabile antilysins from human serum for the Wassermann test consisting of adding 8.2 volumes of $\frac{N}{300}$ hydrochloric acid to 1 volume of heated serum and using the supernatant fluid after the mixture has stood one-half hour followed by centrifugalization to remove the precipitate (see Chapter XXXVIII for details). Uchida[18] has found the method useful and practical in my laboratory for removing anticomplementary substances from human serum but I have found it much less effective for removing the antilysins from rabbit and dog sera.

This suggests that the antilysins of *human* serum are identified with the proteins and very probably with the globulin fractions. Fractionation of anticomplementary human serum with ammonium sulphate has likewise shown that the globulin rather than the albumin fraction carries the antilysins but it would appear that the antilysins are not identified with exactly the same globulins as is the Wassermann antibody since it is possible in Sachs' method to remove a large amount of the antilysins from human serum with but a coincident removal of only a small amount of the complement-fixing antibody.

With *animal* sera as those of the rabbit, dog and ox, it may be that the thermostabile antilysins are more closely associated with the lipoid constituents as suggested by Noguchi; certainly Sachs' method is much less effective for removing their antilysins.

The chemical nature of anticomplementary substances in tissue extracts employed as antigens in the Wassermann reaction has not been accurately determined, but all the evidence indicates that they too are identified with the proteins, as is likewise true of bacterial antigens.

THE MECHANISM OF ANTICOMPLEMENTARY ACTION.

As would be expected in the case of a phenomenon possessing so much academic and practical interest a large number of opinions have been expressed during the past twenty years on the mechanism of the anticomplementary action of sera, spinal fluids and antigens and a few of the more important of these may properly engage our attention.

With the discovery that heating dog, rabbit and some other animal sera at 56° C. rendered them anticomplementary, Ehrlich and Sachs[19] advanced the theory that *complementoids* were produced

which had a greater affinity for sensitized corpuscles than complement and thereby blocked or prevented hemolysis by the latter. But this does not apply to human serum which is not rendered anticomplementary by heating. Nor does it apply to those fresh human and animal sera containing complement and yet anticomplementary both before and after heating. Nor does it apply to normal human spinal fluids because they do not contain enough complement to form complementoid. Furthermore, Miss Yagle and I have observed that while the spinal fluids of normal rabbits and dogs do not contain detectable amounts of complement yet heating them at 56° C. for thirty minutes usually renders them anticomplementary as is true of their sera. In my opinion there is little or no support therefore for the theory and the unmasking of anticomplementary effects of dog and rabbit sera by heat may be explained on the basis that they may contain performed antilysins or that their unheated sera is free of anticomplementary action largely because of the influence of the native complement upon the hemolytic system employed in the tests.

Nor is there any support for the theory of Neisser and Friedemann[20] that the anticomplementary action of serum is due to the development of *amboceptoids* which, when present in excess, are believed to have the power of uniting directly with complement and thereby deviating the latter from union with sensitized corpuscles and preventing hemolysis.

Nor is the anticomplementary action of human, dog, rabbit and other sera to be explained on the basis of the absorption from them by the corpuscles of lipoidal substances, designated by Noguchi as "*protectin*," believed to increase their resistance and thereby resulting in a complete or partial failure of hemolysis. At least Miss Yagle and I have not found sheep corpuscles exposed to human, rabbit and dog sera, previously heated for periods from one-half to three hours at 56° to 58° C., to acquire any increased resistance to either serum or saponin hemolysis; indeed the corpuscles were usually somewhat more susceptible to these agents than the control suspensions.

Nor can the phenomenon be explained on the basis of the formation of *precipitates* with the fixation or absorption of complement, as originally stated by Gay and Moreschi, by a precipitin reaction between the serum or spinal fluid and sheep serum that may adhere to the corpuscles of the indicator antigen; nor by a precipitin reaction between the serum or spinal fluid and the immune rabbit serum carrying hemolysin; nor by a precipitin reaction between the serum and the guinea-pig serum supplying complement; nor by precipitates formed by interaction between the rabbit immune serum and the guinea-pig serum. All of these possibilities have been studied without supporting evidence and it may be also stated in passing that

an anticomplementary serum from a non-syphilitic individual gives no evidences of precipitation with Sachs-Georgi, Kahn or similar antigens.

Trinchese[21] and others have suggested that the phenomenon may be due to the fixation of complement by a *preëxisting antigen-amboceptor complex* or to the development of an antigenic substance in the serum. This theory is difficult to study but Sanford states that he could not elicit any evidence in its support. It is however, deserving of close attention. For example could the apparent anticomplementary state of a fresh serum be due to the fact that it contained an antigen and its antibody in a separated or dissociated state which come together in the test-tube, inactivate the complement and thereby prevent the hemolysis of sensitized corpuscles? Or could a serum contain a natural amboceptor for a bacterium, say the staphylococcus, so that the subsequent contamination of the serum with this organism would furnish an antigen with the formation of an antigen-antibody complex capable of inactivating complement and preventing the hemolysis of sensitized corpuscles?

I have not been able to elicit any clearly-defined evidence in favor of this theory. When a perfectly fresh unheated serum is anticomplementary it is reasonable to assume that a portion or all of its complement is inactivated and this may be readily suspected as due to some profound immunological change the result of disease. Dr. W. E. Gibb has written me that if one first dilutes the serum in the manner of my syphilis complement-fixation method and then adds 0.5 cc. of 1 to 30 complement to each tube followed by incubation for thirty minutes at 37° C., the anticomplementary state may be removed and the complement-fixation tests conducted by heating the tubes for fifteen minutes at 55° C., adding antigen and complement and otherwise finishing the tests in the usual manner. But Miss Yagle and I have found that the addition of 0.5 cc. of saline solution has about the same effects and it is likely that the complete or partial removal of the anticomplementary state under such conditions is really due to the influence of heating at 55° C. upon the diluted serum.

Indeed the exact mechanism is unknown. Complement is extremely susceptible to inactivation and it is definitely known that the antilytic effects of sera, spinal fluids and antigens are due to an injurious influence upon it. When a perfectly fresh unheated serum or spinal fluid is found to be anticomplementary it is entirely likely that it contains no active native complement (although this has not been proven) and that there is something in the serum or spinal fluid capable of inactivating complement; or that some physicochemical change has occurred giving the same result. When a serum or spinal fluid acquires anticomplementary effects it is likely that similar changes in their chemistry are responsible for an inacti-

vation of complement. There is little or no reason to suspect that anticomplementary action is due to a physical absorption or adsorption of complement. In my opinion complement is an enzyme and the anticomplementary action of sera, spinal fluids, antigens and saline solutions is due to an obscure physico-chemical state of these fluids capable of inactivating the enzyme in much the same manner as its inactivation by an antigen-antibody reaction *in vitro* designated as "Complement-fixation."

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PART II.

PRINCIPLES OF COMPLEMENT-FIXATION TECHNIQUE.

CHAPTER VIII.

GLASSWARE AND SALINE SOLUTION FOR COMPLEMENT-FIXATION TESTS WITH SPECIAL REFERENCE TO THE KOLMER REACTION.

Because complement is so susceptible to inactivation it is readily understood that scrupulous care is required in conducting complement-fixation tests to guard against the occurrence of erroneous reactions. This begins with the glassware and saline solution employed and in this chapter the principles are considered while the details of technique of their preparation are given in Chapter XXXI.

PREPARATION OF GLASSWARE.

That variable and erroneous results in the Wassermann and other complement-fixation tests may occur from the use of improperly prepared glassware (test-tubes, pipettes and flasks), mainly due to the presence of traces of acid or alkali, is well known. Doubtless this factor of error is removed in the majority of laboratories by proper methods of cleaning, but the subject is of sufficient importance to demand attention as a fundamental factor in the question of accurate technique, in as much as sufficient amounts of these substances may be present in new glassware or after insufficient rinsing following cleaning with solutions of strong soaps and soap powders, dilute hydrochloric acid or bichromate cleaning fluid, to very materially influence reactions, particularly by a destructive influence upon complement with the danger of falsely positive complement-fixation reactions.

Amounts of sodium hydroxide as small as 0.0002 gm. (1 cc. of 1 to 2000 normal solution) may produce slight hemolysis of washed sheep cells. It is true that the presence of serum affords some pro-

tection but even in its presence 0.0004 gm. (1 cc. of 1 to 100 normal solution) is slightly hemolytic and if present in test-tubes may readily lead to the error of hemolysis and a tendency to falsely negative reactions. Furthermore, and much more importantly, complement is readily inactivated by alkalies and amounts as small as 0.0002 gm. (1 cc. of 1 to 200 normal solution) have been found by Miss Yagle and myself capable of destroying 2 units of 1 to 30 complement. Very small amounts indeed may therefore result in an inhibition of hemolysis due to complement destruction and lead to the error of falsely positive reactions.

But hydrochloric acid sometimes used in the cleansing of test-tubes and pipettes is even more hemolytic and anticomplementary since 0.00007 cc. (1 cc. of 1 to 500 normal solution) may hemolyze washed sheep cells even in the presence of serum while 0.00009 cc. (1 cc. of 1 to 400 normal solution) is capable of destroying 2 units of 1 to 30 complement.

Hemolysin is somewhat more resistant to both agents since 0.002 gm. of sodium hydroxide (1 cc. of 1 to 20 normal solution) and 0.00009 cc. of hydrochloric acid (1 cc. of 1 to 400 normal solution) were found by Miss Yagle and myself to be required for the complete destruction of 2 units of antisheep hemolysin, but even these amounts are quite small. Certainly the results have justified the conclusions of Brown and myself[1] that *these experiments have shown quite conclusively that minute amounts of alkali and mineral acids are markedly antilytic probably by means of a destructive influence upon complement and if present in the test-tubes or other glassware may yield falsely positive Wassermann reactions by reason of their supposedly anticomplementary properties; conversely larger amounts of either may prove hemolytic and thereby yield non-specific or falsely negative Wassermann reactions. For these reasons all glassware used in complement-fixation work must be chemically clean even if not bacteriologically sterile to avoid erroneous results.*

While it is advisable to use sterile glassware this is not absolutely essential but chemically clean glassware and especially test-tubes are essential. Dirty test-tubes may be sterile but contain enough acid or alkali to lead to erroneous results.

In some laboratories it is customary to measure all sera with one pipette, the worker washing out the pipette one or more times by drawing in and expelling saline solution between sera. This would appear from the experiments of Brown and myself as a possible source of error and particularly after pipetting a strongly positive syphilitic serum unless the washing were quite thorough. If serum is drawn up almost to the mouth end and particularly if it touches the cotton of a plugged pipette, the chances of carrying over sufficient positive serum to produce an error are especially present. A further objection is the unavoidable dilution of serum with the

washing fluid adhering to the interior of the pipette, rendering accurate dosage impossible even though the pipette is washed one or more times with the serum before it is measured out (and particularly if the bulk of serum is 1 cc. or less). *For these reasons it is undoubtedly better practice to have a separate pipette for each serum to be tested.*

PREPARATION OF SALINE SOLUTION.

The saline solution employed in complement-fixation tests should (1) be isotonic and otherwise without an injurious effect upon erythrocytes; (2) contain the proper amounts of salts essential for serum hemolysis and (3) be free of anticomplementary effects, that is, free of substances capable of inactivating or destroying complement. It is ordinarily an 0.85 per cent solution of sodium chloride in distilled water although serologists have used from 0.7 to 1 per cent solutions; the average of 0.85 per cent is usually preferred and has been designated as *isotonic* or *physiological saline solution*. Solutions containing less than 0.7 per cent sodium chloride are hypotonic and hemolytic; those containing more than 1 per cent are hypertonic and apt to be antihemolytic or feebly hemolytic in high concentrations. For example, Miss Yagle and I have observed that 2 to 10 per cent solutions inactivate complement and thereby prevent hemolysis while 25 per cent solutions are distinctly hemolytic, that is, capable of destroying erythrocytes directly. It may be stated in passing however, that 1 to 25 per cent solutions do not prevent the union of antisheep hemolysin with washed sheep corpuscles, although it is sometimes stated that hypertonic solutions may prevent sensitization.

Otherwise, very little attention has been given to this subject and I first thought that the preparation of saline solution would be one of the least important in relation to the standardization of the complement-fixation test for syphilis; but experience has since shown that the importance of the subject was underestimated insofar as my test is concerned, since the complement is used in a dilution of 1 to 30 which renders it more susceptible to the destructive influences of impurities, as well as to fluctuations in both salt and hydrogen-ion concentrations, than is the case in tests using lower dilutions of complement serum like 1 to 10 or 1 to 20.

Choice of Salt for Saline Solution.—As a general rule chemically pure sodium chloride is to be preferred in the preparation of saline solution for the complement-fixation test, although Brown and I have found that ordinary table salt of good quality may be satisfactory. The former however, is to be preferred because a crude product may introduce impurities capable of rendering the solution either hemolytic or anticomplementary as well as altering the optimum salt and hydrogen-ion concentrations. Probably the amount

of magnesium present is of special importance as indicated by the work of Kellogg and Wells, shortly to be discussed with more detail. Compressed tablets of sodium chloride may be unsatisfactory.

Optimum Amount of Salt.—The important relation of salt to the physical chemistry of the complement-fixation reaction has been already referred to in Chapter VI. It must be sufficient for isotonicity but the question of the best amount to employ in relation to the mechanism of serum hemolysis and complement-fixation has not attracted much attention.

Most serologists employ 0.85 to 1 per cent solutions but Neukirch[2] states that 0.72 per cent solutions give stronger Wassermann reactions with weakly positive syphilitic sera and that sera yielding negative reactions in tests employing 0.85 per cent saline solution may give true positive reactions with 0.72 per cent solutions.

In my experiments 0.7 per cent solutions were sometimes slightly hemolytic and yielded a high percentage of anticomplementary serum controls; 1 per cent solutions were sometimes slightly anticomplementary and likewise undesirable when 1 to 30 complement was employed. Solutions of 0.72 per cent were not hemolytic for fresh corpuscles (but sometimes slightly so for preserved blood) and in a series of comparative tests with 0.85 per cent solutions by Miss Yagle, Miss Richter and myself it was found that they were likewise particularly prone to yield anticomplementary reactions; that is, the serum controls were incompletely hemolyzed or non-syphilitic sera failed to give completely negative reactions, although the sera were fresh and free of all anticomplementary effects in the tests employing 0.85 per cent solutions. Of course under these conditions the positive reactions with the 0.72 per cent solutions were sometimes stronger than with the 0.85 per cent solutions but in no instance have sera tested with the 0.72 per cent solutions given true positive reactions with negative reactions in the control tests conducted with the usual 0.85 per cent solutions. Therefore, I am unable to endorse the use of 0.72 per cent saline solution in the Wassermann test at least. It is true that when used the units of hemolysin and complement may be reduced and especially when formalized sheep cells are used but this is no real advantage. Even when the units of hemolysin and complement are the same as with 0.85 per cent solutions, tests conducted with 0.72 per cent saline show a tendency to an increased percentage of anticomplementary reactions or incomplete hemolysis of negative controls, probably because of an insufficient amount of salt for the mechanism of serum hemolysis.

Choice of Water for Saline Solution; Buffered Saline Solutions.—This is probably a more important subject than the chemical purity of the sodium chloride. Dr. Brown and I observed that in Philadelphia saline solution could be prepared of either distilled or raw (tap)

water but advised the use of the former. But soon after the introduction of my test reports began to reach me of difficulties being experienced in the titration of hemolysin and complement. For example, antisheep hemolysin with a unit of 0.5 cc. of 1 to 8000 or higher in my laboratory were reported as low as 0.5 cc. of 1 to 1000; naturally one first suspected that the trouble was due to a defective complement serum. In some instances this was the case but in the majority it was found that the saline solution was anticomplementary. Curiously enough in every instance so far reported to me the trouble was due to the use of distilled water and was promptly corrected by the use of raw or tap water for preparing saline solution.

Mason and Sanford[3] have observed that saline solutions prepared with distilled water were more sensitive to changes in hydrogen-ion concentration as the result of boiling or the addition of carbon dioxide than solutions prepared with tap water and that the latter were less likely to become acid by reason of the presence of natural buffers. In their opinion the optimum range should be in the zone of pH 7.0 to 8.4 and since it is impossible to prepare a standard saline solution using tap water, because its composition varies in different localities, or a solution of definite hydrogen-ion concentration from unbuffered distilled water, they have suggested the use of the following buffered saline solution as one having a definite and constant composition:

Sodium chloride	7.0 gm.
Secondary sodium phosphate (2 molecules of water)	1.7 gm.
Primary potassium phosphate	0.2 gm.
Double distilled water	1000.0 cc.

This combination yields a solution with a pH of 7.4 to 7.8 and a salt concentration of 8.9 gm. per liter. In their experience it has yielded uniform and constant results, and Rockwood[4] states that while it undoubtedly stabilizes a variable factor in the reaction, it does not act by buffering the final mixture to neutrality since the buffer value of the cells is already sufficient for accomplishing this, except in extreme instances.

Unquestionably the pH of saline solution is a matter of practical importance but I doubt if it is the sole factor at issue. My saline solutions prepared of either distilled or tap water have a pH of about 7.4 but I have found that solutions prepared of distilled water sent to me by serologists from different parts of the United States may still prove anticomplementary in spite of the pH being 7.4 to 7.8, whereas solutions prepared of tap water and having the same pH range were perfectly satisfactory. In my experience the buffered solution of Mason and Sanford is somewhat hemolytic and when made up with distilled water has not proven as satisfactory as when prepared with Philadelphia tap water although the range of pH

for both was 7.3 to 7.4. In other words *the natural buffers of tap water are apt to be more satisfactory in relation to serum hemolysis than artificially buffered distilled water.*

Unfortunately I am not able to discuss the chemical composition of tap water in relation to this subject; Kellogg and Wells[5] however, have recently observed that the amount of magnesium present in water or salt is a matter of importance. Of course the magnesium content may vary during the year but probably not within limits to influence complement activity; at least seasons have no influence upon Philadelphia tap water insofar as its use on saline solution is concerned. Mr. Herman Brown, Chemist to the Research Institute of Cutaneous Medicine, has found that the magnesium of our water calculated as $MgCl_2.6H_2O$ averaged 0.0835 gm. per liter which is very near the 0.1 gm. per liter found by Kellogg and Wells as optimum for saline solution; the calcium (which largely determines the hardness) averaged 0.0785 gm. per liter calculated as $CaCl_2.H_2O$ and the results of general chemical analyses as reported to me by the Water Bureau have been approximately as follows (to obtain grains per gallon divide by 17.1):

Total solids	79–198
Nitrogen as total organic and Ammonium	0.10–0.29
Nitrites	0.000–0.000x
Nitrates	0.79–1.52
Oxygen consumed	1.1–1.6
Chlorides	5.6–8.9
Iron	0.06–0.11
Sulphates (SO_3)	20–67
Alkalinity	29–41
Total hardness (soap)	45–107
Permanent hardness	16–66
Carbon dioxide	6–7

Very probably an excess of lime salts may render tap water unsatisfactory and require the use of a distilled water in some localities but I believe that *whenever difficulties are experienced with hemolysin or complement titrations one should first suspect the saline solution as being responsible and if prepared of distilled water, to substitute a solution prepared of ordinary tap or spring water and chemically pure sodium chloride.* As Dr. C. M. Anderson, Director of Laboratories of the Provincial Department of Health of Ontario has written me, "The titers of complement and hemolysin are higher and the complement-fixation reactions slightly stronger with saline solution prepared of tap water and for several years I have used saline solutions prepared of tap water and chemically pure sodium chloride with perfect results."

But if saline solutions prepared with tap or distilled waters and chemically pure sodium chloride are still unsatisfactory, it may be well to add 0.1 gm. magnesium sulphate or chloride to each liter as suggested by Kellogg and Wells, who state:

"We have performed many experiments with various lots of sodium chloride and have included variations in the pH values as well as slight variations in the tonicity of the solutions; tap, distilled, and double distilled waters being used. There is nothing to be gained in tabulating these experiments as nothing definite resulted beyond additional confirmation of the idea that reaction in itself was relatively unimportant. One particular lot of salt gave such uniformly high titrations that it was decided to search for a chemical difference between this and other samples. The outstanding characteristic of this brand of salt was found to be the presence of considerable amounts of magnesium. The next step was to analyze the tap water which also gave high titrations with chemically pure sodium chloride, and it was found to contain 14 parts per million of magnesium. This quantity, estimated as chloride, represented 0.12 gms. per liter. Experiments with physiological saline solution made with chemically pure salt, distilled water and with varying amounts of magnesium chloride showed that less than 0.02 gms. per liter were without effect or had little effect on the titration, but that 0.05 and over markedly raised the titer of amboceptor over that obtained with salt solution not containing magnesium. The upper limit for the addition of magnesium chloride was reached, when a mixture of isotonic sodium chloride solution and a mixture of isotonic magnesium chloride (19 grams of moist crystals per liter) in the proportion of 98 parts of the former and 2 parts of the latter was used. Any further addition of magnesium beyond this point resulted in a rapid fall in the titer of amboceptor until absolute inhibition of hemolysis was reached with 5 parts of the magnesium solution to 95 parts of normal salt solution."

I have not had the opportunity of trying out this suggestion under proper circumstances because both tap and distilled water have been uniformly satisfactory in my laboratory. We have observed however, that while the addition of magnesium sulphate or chloride did not improve our saline prepared of tap water that it slightly improved that prepared of distilled water, as shown in the following results of an experiment employing 0.85 per cent solutions of chemically pure sodium chloride, with and without the addition of 0.1 gm. per liter of $MgSO_4$ or $MgCl_2$:

	Unit of hemolysin 0.5 cc. of	Unit of complement 1 to 30
Plain saline prepared of distilled water . .	1 to 3000	0.45 cc.
Saline prepared of distilled water plus $MgSO_4$	1 to 6000	0.40 cc.
Saline prepared of distilled water plus $MgCl_2$	1 to 6000	0.40 cc.
Plain saline prepared of tap water	1 to 6000	0.30 cc.
Saline prepared of tap water plus $MgSO_4$ . .	1 to 4000	0.45 cc.
Saline prepared of tap water plus $MgCl_2$ . .	1 to 5000	0.40 cc.

It would appear therefore that nothing is to be gained by adding magnesium to a satisfactory saline solution, nor have Kellogg and Wells advised it, but when a saline prepared of distilled or tap water and chemically pure sodium chloride is unsatisfactory I believe it well worth while to try the addition of 0.1 gm. magnesium sulphate or chloride to each 1000 cc. as they recommend.

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CHAPTER IX.

CHOICE OF A HEMOLYTIC SYSTEM FOR COMPLEMENT-FIXATION TESTS.

In conducting the Wassermann and other complement-fixation reactions, washed erythrocytes and their hemolysin are added at the end of the period of primary incubation and the mixtures reincubated as a test for the presence of free or active complement. The test corpuscles, hemolysin or amboceptor and complement constitute the *hemolytic system* and following the original Wassermann method is usually composed of washed sheep erythrocytes, antisheep hemolysin and guinea-pig complement.

Practical Importance of the Subject.—But other systems have been advocated like the antiox, antihuman, antichicken, antigoat, etc. The choice is sometimes determined by the availability and convenience of securing the blood cells but the matter is far too important to be decided upon by convenience only, since the kind of hemolytic system employed influences in an important manner the sensitiveness and even the specificity of the Wassermann and other complement-fixation reactions.

For example, the question of natural hemolysins in patients' sera is of some importance and especially in relation to the antisheep system because weakly positive Wassermann and other complement-fixation reactions may be "missed" or undetected when an excess of hemolysin "makes up" for a slight degree of fixation or inactivation of complement by antigen and antibody and thereby yields a falsely negative reaction of complete hemolysis.

Likewise the matter of natural hemolysins in the complement serum requires consideration for the same reason although in my test this factor is automatically and routinely adjusted by the practice of titrating the immune hemolysin before the main tests are conducted; if the complement happens to contain natural hemolysin just so much less immune hemolysin is required.

The question of natural hemagglutinins in patients' sera and complement is also of practical significance since agglutination of the test erythrocytes interferes with hemolysis and is a fertile cause of doubtful reactions.

But the most important subject of all is the strength or titer of the immune hemolysin. Powerful amboceptors like the antisheep and antiox, which ordinarily yield units of 0.5 cc. of : 4000 or higher

in my method, introduce into the test-tubes the minimum of rabbit serum and, what is far more important, permit the tests to be conducted with the smallest amounts of complement which greatly increases the sensitiveness of the Wassermann and other complement-fixation reactions. Furthermore, when such powerful hemolysins are used, interference with hemolysis by the hemagglutinins in the hemolysin serum as well as in the patient's serum and complement, is prevented. Equally powerful antihuman and antichicken hemolysins cannot be ordinarily and routinely prepared by the immunization of rabbits or other animals and consequently their use requires relatively larger amounts of complement to complete the hemolytic system, which experience has shown reduces the sensitiveness of the reactions. Furthermore, the hemagglutinins so frequently present may interfere with hemolysis and greatly complicate the readings.

So it is readily apparent that the choice of a hemolytic system is an important practical subject requiring close study from many different angles which it is my purpose to discuss at this time along with the reasons for adopting the antisheep or antiox systems as those of first choice.

INFLUENCE OF NATURAL HEMOLYSINS UPON THE COMPLEMENT-FIXATION REACTION.

The most frequently expressed objection to the antisheep hemolytic system is the presence of variable amounts of natural hemolysin in human sera which may so disturb quantitative relationships that falsely negative reactions of complete hemolysis sometimes result.

Natural Hemolysins in Human Sera.—Human sera contain a surprisingly large number of different hemolysins and especially in the fresh unheated state. After heating at 55° C. for fifteen to thirty minutes the incidence is sharply reduced although natural antisheep hemolysin is much more thermostabile than the others. Figures expressing percentages of natural hemolysins vary a good deal according to the technique employed and especially in relation to the amount of serum tested and the amount of complement employed. Furthermore, all of the natural hemolysins in human sera and especially the thermolabile ones, are arranged in types or groups as observed by Trist, Flick and myself,[1] analogous to the four main groups in human sera; the percentages vary therefore according to whether the corpuscles of one or several animals of the same species are employed in the tests.

In my complement-fixation method employing 0.3 cc. of 1 to 30 complement and 0.5 cc. of 2 per cent corpuscles about 85 per cent

of human sera heated at 55° C. for fifteen minutes and tested in 0.1 cc. amounts show the presence of antisheep hemolysin. In about 50 per cent enough is present to give complete hemolysis while in the balance of about 35 per cent partial hemolysis occurs; similar results were observed in a prior study by Casselman and myself.[2] Table 4 gives the average results:

TABLE 4.

Kind of hemolysin.	In unheated serum, per cent.	In heated serum, per cent.
Antihuman	4 to 44	0 to 4
Antisheep	85 to 95	80 to 95
Antiox	72 to 88	5 to 30
Antiguinea-pig	90 to 100	20 to 25
Antirabbit	94 to 100	0 to 2
Antihog	92 to 100	0 to 15
Antirat	4 to 32	0 to 2

It is apparent therefore, that if unheated patient's serum is employed it is almost impossible to avoid natural hemolysins and some like those for sheep and guinea-pig corpuscles occur so frequently that complement-fixation tests have been devised for utilizing them along with the native complement in conducting complement-fixation tests, as in the methods of Hecht, Weinberg, Gradwohl, Tschernogubow and others. As shown by Williams[3] using Group IV human corpuscles (Moss), corresponding to Group I of Jansky, makes it possible to avoid the influence of natural antihuman hemolysins in human and guinea-pig sera and this is certainly the hemolytic system of first choice when unheated serum is employed.

With heated serum the antisheep system would appear to be the least desirable because fully 85 per cent of sera retain some natural hemolysin. The antiox system is better in this respect while the antihuman and antichicken systems would be very much better except for the fact that it is so difficult to prepare immune hemolysins comparable in high potency to those readily prepared for sheep and to lesser extent for cattle cells.

Natural Hemolysins in Guinea-pig Sera.—Since guinea-pig serum is the complement of choice, Matsunami, Trist and myself[4] have studied a large number for natural hemolysins and found that approximately 40 per cent contained some antisheep, 32 per cent antiox, 40 per cent antichicken and 5 per cent antihuman hemolysins. But the presence of a natural hemolysin in the complement serum for the test corpuscles is of no practical importance providing the immune hemolysin is titrated with the complement preliminary to the complement-fixation tests, as is done in my method. If the complement should happen to contain natural hemolysin so much less immune hemolysin is required. For example the unit of antisheep hemolysin titrated with 0.3 cc. of 1 to 30 hemolysin-free complement was 0.5 cc. of 1 to 6000; when titrated with a comple-

ment containing a good trace of natural antisheep hemolysin the unit was 0.5 cc. of 1 to 8000, which meant that less immune hemolysin was required. For this reason I believe that it is always well to take the little extra time required for titrating the hemolysin with each complement instead of using the hemolysin in a constant and fixed amount from week to week; furthermore, with this method it is unnecessary to test the complement serum for natural hemolysin.

Influence of Natural Antisheep Hemolysin upon the Wassermann and Other Complement-fixation Reactions.—The influence of natural antisheep hemolysin upon complement-fixation reactions varies a good deal according to the method employed. For example, the effects are more pronounced in the original Wassermann test conducted with 0.2 cc. of patient's serum than in some modifications employing smaller amounts of serum and a more closely adjusted hemolytic system. Furthermore, the effects are more pronounced when the tubes are allowed to stand over night in a refrigerator than when the reactions are read within an hour or two after the secondary incubation.

A very large number of comparative tests with syphilitic sera before and after the removal of natural antisheep hemolysin have shown that in my method about 2 to 5 per cent more sera give a true positive reaction after removal and about 6 per cent give stronger reactions. These were approximately the figures arrived at by Miss Rule and myself[5] several years ago and are essentially similar to those observed in hundreds of additional comparative tests conducted since that time. Kilduffe[6] and Uchida[7] have reported slightly higher figures indicating that as many as 8 to 10 per cent of syphilitic sera reacting negatively before removal of natural antisheep hemolysin may yield true positive reactions after removal. But these figures may be a little too high insofar as clear-cut positive *versus* negative reactions are concerned although correct if including those sera showing stronger reactions after removal of hemolysin.

Sera containing large amounts of antibody are not materially influenced by the presence of natural hemolysin; sera containing small amounts of complement-fixing antibody and large amounts of natural hemolysin are the ones likely to give falsely negative reactions unless the natural hemolysins are removed. This is possibly true in my test conducted with a single dose of 0.1 cc. of serum and may account for the fact that this amount of serum may give a weaker Wassermann reaction than 0.05 cc. of serum; partly for this reason I now conduct my qualitative test with 0.1 cc. and 0.05 cc. of serum instead of with 0.1 cc. only as formerly described. With strongly positive syphilitic sera one must add a very large excess of hemolysin before falsely negative reactions are produced but with weakly positive

sera much less hemolysin will produce this result and especially if the tubes are placed in a refrigerator over night before the readings are made which permits the excess of hemolysin to continue hemolysis after the secondary incubation.

It is apparent, therefore, that if one strives for the maximum degree of sensitiveness consistent with reliable and specific complement-fixation reactions, the natural antisheep hemolysin should be routinely removed from all sera before they are heated and tested. This has been my practice for over two years and I believe that the results justify the extra labor involved. Furthermore, the reactions should be read at the end of the secondary incubation but if the natural hemolysins are not removed beforehand and if the tubes are allowed to settle over night the influence of excess hemolysin may be prevented by inactivating the remaining complement by heating the tubes at 55° C. for ten minutes before placing them in the refrigerator, as described by me several years ago.[8]

Methods for Removal of Natural Antisheep Hemolysin.—The method of removal of natural antisheep hemolysin has an influence upon the results. For several years I have added about 1 drop of washed sheep corpuscle sediment to approximately each cubic centimeter of *unheated* serum *on the clot*, followed by thorough mixing, refrigerator for about fifteen minutes, centrifuging, and heating the serum at 55° C. for fifteen minutes. This removes the induced anticomplementary substances in almost all sera. For example, syphilis complement-fixation tests with 1000 sera by my method showed that 3 per cent were anticomplementary; after absorption of the antisheep hemolysin only 3.6 per cent were anticomplementary which is a negligible increase. If absorption of the serum is carried out for thirty to sixty minutes in the refrigerator the incidence of anticomplementary reactions increases in my experience to about 7.5 per cent and at room temperature for thirty minutes to about 13 per cent. Absorption in the refrigerator over night gave as high as 40 per cent anticomplementary sera in one series of tests on 1115 sera. If, however, the sera are first heated and then absorbed a larger number are anticomplementary and this may account for apparently stronger reactions even though the serum controls show complete hemolysis.

The method of removal of antisheep hemolysin briefly described (the details are described in Chapter XXXI) has been found to absorb *all* of the hemolysin in 96 to 97 per cent of sera; a mere trace may escape removal in occasional sera containing excessive amounts. I have also tried to absorb the hemolysin by adding to each cubic centimeter of serum approximately 0.05 gm. of dried sheep blood prepared by washing the corpuscles, hardening them by exposure to 1 to 100 formalin in saline over night in a refrigerator, centrifuging and drying the sediment rapidly in a vacuum. The mass was then ground into a fine powder and stored. This material removed the

antisheep hemolysin from about 85 per cent of sera after absorption in a refrigerator for thirty to sixty minutes. Sometimes the sera were deeply tinged with hemoglobin. I had hoped to be able to remove natural antisheep hemolysin routinely from all sera by placing the powder in test-tubes before collection and then mixing well with the blood before coagulation occurred. Unfortunately however, it did not work as well as removal of the hemolysin by adding fresh washed sheep corpuscles as described above and I know of no substitute for this method, as adsorption with barium sulphate and such agents has failed to remove hemolysin without removal of syphilis or other kinds of complement-fixing antibody.

A Comparative Study of Complement-fixation in Syphilis with Antihuman, Antiox, Antichicken and Antisheep Hemolytic Systems.— Since heated human sera contain so much less natural antihuman and antichicken than antisheep and antiox hemolysins, the influence of natural hemolysins upon the Wassermann and other complement-fixation reactions can be studied by comparative tests with these systems.

Indeed a great deal has been written upon the comparative results of complement-fixation tests conducted with an antisheep system after the method of Wassermann and an antihuman system after the method of Noguchi. Unfortunately much of this work is not strictly comparative or adapted for critical analysis inasmuch as the technique of the two methods usually varied in one or more essential details, among which may be mentioned whether the sera were heated or unheated, and whether the primary incubation was conducted in a thermostat or in a water-bath; these factors have an important bearing upon the results and in strictly comparative tests the technique should be identical in all details. Furthermore, much of the work reported in literature was conducted with one kind of antigen in the Wassermann tests and a different antigen in the Noguchi tests, and the tremendous influence exerted upon the results of any method by the kind of antigen employed is well known; likewise the investigations of Morgenroth and Sachs[9] have shown that the activity of hemolysins bears a close relationship to the amount of complement in the hemolytic system, and for this reason tests for the determination of the influence of natural antisheep hemolysin in human sera upon complement-fixation based upon comparative results with antisheep and antihuman hemolytic systems should be conducted with similar amounts of complement in the hemolytic system.

In an investigation of this kind by Matsunami, Rule and myself,[10] a large number of *syphilitic* sera were subjected to comparative tests with antisheep, antiox, antichicken and antihuman hemolytic systems. By using these we have attempted an accurate determination of the influence of natural hemolysins in human and guinea-pig

sera (complement) and particularly that for sheep cells, upon the results of complement-fixation reactions and have sought to arrive at a decision upon the important question regarding the best hemolytic system for a standardized complement-fixation test.

The results have shown that in tests conducted with 0.1 cc. amounts of heated sera and a cholesterolized alcoholic extract of heart antigen, the antihuman and antichicken systems yielded from 1 to 4 per cent more positive reactions than the antisheep and antiox systems. The antiox system yielded from 2 to 5 per cent more positive reactions than the antisheep. But since then a more recent study by Miss Yagle and myself has shown however, that the difference may not be as much since the antiox system gave but 1.5 per cent more positive reactions and *additional comparative tests employing sera from which the natural antisheep hemolysin was removed have shown that the antisheep system has actually yielded a higher percentage of true positive reactions than the antiox, antichicken and antihuman systems.* I believe that this result is due to the fact that the antisheep system in my test utilizes 1 to 30 dilution of complement whereas the antiox usually requires 1 to 20 because the hemolysin cannot be prepared of equal titer while the antihuman and antichicken systems require 1 to 5 or 1 to 10 complement because of the weaker hemolysins. Now the minimum amounts of complement and immune hemolysin (which introduces the smallest amounts of guinea-pig and rabbit sera into the complement-fixation test) increase the sensitiveness of the reactions and accounts for the fact that when my *quantitative* test is conducted with the antisheep and antiox systems (even without removal of natural hemolysins from the sera), the reactions are slightly more sensitive than with the antihuman and antichicken systems.[11] When the natural antisheep hemolysin is removed from the sera, the quantitative reactions with the antisheep system are much more sensitive.

A Comparative Study of Complement-fixation in Bacterial Diseases with Antisheep, Antiox and Antihuman Hemolytic Systems.—Similar comparative complement-fixation tests employing tuberculosis, gonococcus, glanders and typhoid antigens in one-third anticomplementary units and their respective immune sera in graded amounts in order to elicit the finer degrees of variation in degree of complement-fixation, were conducted at the same time and with the same guinea-pig complements with the following hemolytic systems: antisheep, antiox and antihuman. Owing to variation in the potency of the rabbit immune hemolysins, it was necessary to dilute the complement 1 to 30 in the antisheep system; 1 to 20 in the antiox and 1 to 10 in the antihuman system. Otherwise the technique was identical in all tests.

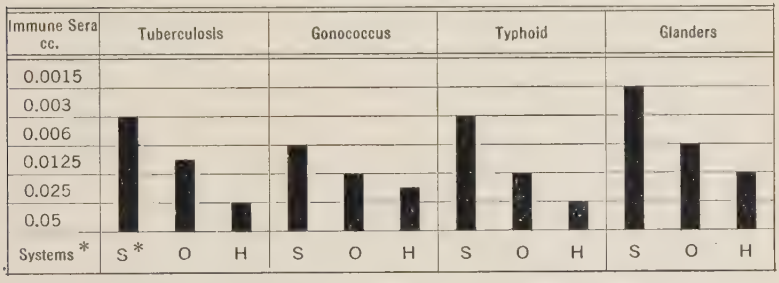
The results of one experiment are shown in Fig. 1 and the study may be summarized as follows:

1. The antisheep system yielded the most sensitive reactions.
2. The antiox system and antihuman system yielded slightly less sensitive reactions.

These differences were not apparent in the tests carrying the larger amounts of the respective immune sera (0.05 cc.) but only in the higher dilutions and smaller amounts.

The only reason I can assign for these differences refers to the complement serum, that is, the higher the dilution and the smaller the amount employed the more sensitive are the reactions. Owing to the impossibility of preparing antihuman hemolysin of equal potency to antisheep, it is necessary to use more complement with the antihuman system and fixation is less complete. For example, in the tests summarized in Fig 1 the amounts of complement (*2 full units*) employed with the respective hemolytic systems were as follows:

Antisheep system: 0.7 cc. 1 to 30 = 0.023 cc. undiluted serum.
Antiox system: 0.6 cc. 1 to 20 = 0.033 cc. undiluted serum.
Antihuman system: 0.3 cc. 1 to 10 = 0.033 cc. undiluted serum.



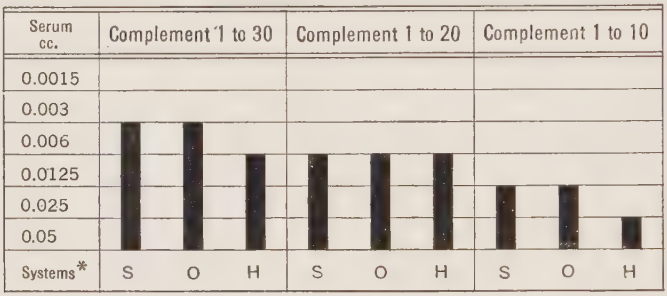
* S = antisheep; O = antiox; H = antihuman.

FIG. 1.—Comparative sensitiveness of different hemolytic systems in bacterial complement-fixation.

As previously stated, the technique of these comparative tests was otherwise absolutely identical, conducted with the same mixture of guinea-pig complement sera and at the same time. A series of similar tests showed exactly the same results; namely, that the three hemolytic systems did not show identical results and that in the order of sensitiveness of reactions the systems were as follows: antisheep, antiox, and antihuman.

However, when more powerful antiox and antihuman hemolysins are available permitting the use of 1 20 and 1 to 30 dilutions of complement, the reactions are of the same sensitiveness or almost so as observed with the antisheep system as shown by the results of tuberculosis complement-fixation in Fig. 2. In other words tests conducted with the antisheep and antiox systems employing 1 to 30

dilutions of the same complement according to the technique of my new test have yielded practically identical reactions; usually (but not always) tests conducted with the antihuman system have yielded slightly less sensitive reactions. Tests conducted with 1 to 30 complement in all three hemolytic systems have yielded more sensitive reactions than duplicate tests conducted with 1 to 20 complement and tests conducted with 1 to 20 complement have yielded more sensitive reactions than tests employing 1 to 10 complement. These facts are shown graphically in Fig. 2 and indicate that *the most sensitive complement-fixation reactions are to be observed when high dilutions of complement are employed with powerful hemolysins.*



* S = antisheep system; O = antiox system; H = antihuman system.

FIG. 2.—The influence of amount of complement serum upon the sensitiveness of the tuberculosis complement-fixation reaction.

THE INFLUENCE OF HEMAGGLUTININS UPON THE COMPLEMENT-FIXATION REACTION.

In the antisheep hemolytic system the hemagglutinins have no effect at all if hemolysin of the usual and ordinary titer is employed. The same is true of the antiox system.

But in the antihuman and antichicken systems the hemagglutinins may be very troublesome and by causing agglutination of the erythrocytes interfere with complete hemolysis and thereby complicate the reading and interpretation of weakly positive reactions. And I may state again as a firm conviction based upon extensive clinical and serological experience especially in syphilis, that any technique ignoring weakly positive reactions because their specificity is questioned, fails as an acceptable method.

Hemagglutinins in relation to complement-fixation may occur in the patient's serum being tested and in the immune rabbit serum employed as hemolysin or amboceptor. The latter is probably the most important since large amounts of agglutinin may be produced by the immunization of rabbits and especially with human and chicken erythrocytes. As shown by Matsunami, Trist and myself

unheated guinea-pig complement is devoid of agglutinins for sheep, cattle, human, chicken and guinea-pig erythrocytes and this possible source may be disregarded; heated guinea-pig sera may contain agglutinins but this is of no particular interest because only fresh unheated sera are employed in complement-fixation tests.

Natural Hemagglutinins in Human Sera.—Just as human sera contain many different natural hemolysins so likewise many hemagglutinins are to be found and especially in microscopic tests. As is now well known the agglutinins for human erythrocytes (isohemagglutinins) occur in four main serological groups and similar groups occur for the erythrocytes of the lower animals as shown by Trist, Flick and myself. The percentages therefore, vary within rather wide limits according to the corpuscles employed and Table 5 gives the average results observed in tests employing 0.1 cc. of heated sera:

TABLE 5.

Erythrocytes.	Percentages.
Human	4 to 80
Sheep	4 to 36
Ox	4 to 40
Guinea-pig	24 to 36
Rabbit	90 to 100
Rat	66 to 85
Hog	64 to 90
Dog	About 88
Chicken	0 to 4

It will be observed that agglutinins may be present for the corpuscles of the four chief hemolytic systems, namely, the sheep, cattle, human and chicken. But I have never seen the slightest evidence of agglutination in the sheep system because the hemolysin is ordinarily so powerful that complete hemolysis occurs; the same is usually true of the antiox system. In the antihuman system however, agglutination is usually well marked unless the tests are conducted with relatively large amounts of complement and sufficiently powerful hemolysin; the same is true of the antichicken system.

Hemagglutinins in Immune Rabbit Serum.—When rabbits are immunized with washed sheep corpuscles after my method and titrated in the presence of 0.3 cc. of 1 to 30 complement and 0.5 cc. of 2 per cent cells the unit of hemolysin is usually between 0.5 cc. of 1 to 4000 to 1 to 10,000; the agglutinin content is usually not higher than 1 to 300. Somewhat similar conditions apply to the antiox system so that agglutination of the test corpuscles is never apparent in my test when the usual antisheep and antiox hemolysins are employed.

But it is almost impossible to avoid the influence of the agglutinins in antihuman and antichicken hemolytic sera. They are almost

invariably present and especially in the former, although with exceptionably powerful sera their influence may be removed. Nor can the influence of immune agglutinin for human erythrocytes be prevented by selecting Group IV erythrocytes (Moss) because rabbits injected with human erythrocytes belonging to any one of the four serological groups produce agglutinins for all four groups as shown by Trist and myself.[12] Nor has any method been devised for removing the agglutinins without coincident removal of the hemolysins, as shown by my former students, Sands and West.[13] Nor can there be any denial of the fact that agglutination of the test erythrocytes may interfere with complete hemolysis and thereby render difficult or impossible the interpretation of the results of weakly positive complement-fixation reactions. Indeed this difficulty constitutes one of the most important reasons for selecting the antisheep or antiox systems as the ones of choice in conducting complement-fixation tests possessing the maximum degrees of sensitiveness and specificity and especially quantitative reactions employing varying amounts of patient's serum.

CHOICE OF HEMOLYTIC SYSTEM.

From the standpoints of sensitiveness and specificity I believe therefore, that the antisheep and antiox hemolytic systems are the ones of first choice. It is true that these erythrocytes may not be available in some localities and especially on board ships but under such very exceptional circumstances the antihuman system may be used and especially since corpuscles may be obtained by breaking up clots of blood submitted for the complement-fixation test.

Blood for the antisheep or antiox systems may be obtained from an abattoir and since it is an easy matter to preserve the cells with formalin, a supply every two or three weeks is sufficient. It is not at all necessary to maintain one or more sheep as a source of blood cells.

As I shall discuss with more detail in Chapter XI guinea-pig complement is the one of first choice. It was first employed by Bordet and Gengou in 1901, was adopted by Wassermann and his colleagues in 1905 and has ever since been the complement of choice. Of course the sera of human beings and many of the lower animals have been tried but from every standpoint including that of high hemolytic activity, fixability by antigen and antibody, freedom from unusual sensitiveness to anticomplementary effects of serum alone and antigen alone, freedom from natural hemolysins, bacterial amboceptors, etc., as well as from the standpoint of convenience and availability, a mixture of guinea-pig sera has proven best as a source of complement. Furthermore it can be preserved over several weeks

which is a distinct advantage for laboratories employing small amounts.

Human serum ranks next in choice and is actually more susceptible to fixation by antigen and antibody but it is at least seven times weaker in hemolytic activity and it is more susceptible to non-specific effects.

Antisheep hemolysin or amboceptor originally employed by Wassermann, Neisser and Bruck continues to be the one mostly employed. It is easy to prepare by the immunization of rabbits with washed sheep erythrocytes, that is to say, high titered immune sera are readily produced and these enable one to conduct the Wassermann and other complement-fixation tests with minimal amounts of the immune serum and complement which, as stated above, contributes in an important manner to sensitive and specific reactions. The one drawback to its use is the fact that so many human sera contain natural antisheep hemolysin and when this is present in unusually large amounts the excess tends to reduce the sensitiveness of the reactions. But it is an easy matter to remove the natural hemolysins routinely and by a close adjustment of the hemolytic system along with the practice of reading the reactions immediately after the secondary incubation, their effects upon the reactions may be entirely removed.

Antiox hemolysin ranks second in choice and indeed if beef blood were as easily obtainable as sheep blood and if rabbits could be made to yield higher titered hemolytic sera than is usually the case, it would rank ahead of antisheep hemolysin because human sera contain much less natural antiox hemolysin.

Antihuman hemolysin has had many advocates because the influence of natural hemolysin upon the reactions is greatly reduced. But human and guinea-pig sera may also contain natural hemolysins for human erythrocytes unless Group IV corpuscles (Moss) are employed, which may likewise exert an influence upon the reactions. But the great drawback to the use of antihuman hemolysin is the fact that high titered sera cannot be ordinarily produced and for this reason much more complement is required for complete hemolysis which reduces the sensitiveness of complement-fixation reactions. Furthermore, antihuman hemolysin always contains agglutinins for human erythrocytes which may interfere with complete hemolysis and greatly confuse the reading and interpretation of weakly positive complement-fixation reactions, which should always engage the serious attention of serologists, since to ignore them greatly reduces the real value of the complement-fixation reaction in syphilis as an aid in diagnosis and a guide in treatment.

The same objections pertain to antichicken hemolysin with the added disadvantage that the nuclei of hemolyzed corpuscles collecting in the bottom of the test-tubes sometimes add to the confusion

in reading and interpreting the important weakly or doubtfully positive Wassermann reactions. For these reasons I have adopted *the antisheep system as the one of first choice* with the antiox second and the antihuman a poor third.

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CHAPTER X.

RED CORPUSCLE SUSPENSION FOR THE COMPLEMENT-FIXATION TEST; THE PRESERVATION OF ERYTHROCYTES.

Comparatively little attention has been given to various methods for the collection of blood and preparation of a suspension of washed erythrocytes for the indicator antigen in complement-fixation tests. A few points however, are worthy of some attention from the standpoint of accuracy and uniformity of technique.

Sources of Blood.—When the sheep hemolytic system is employed, an animal or two may be kept by the laboratory and bled from a vein as required according to the methods shortly to be described, but in cities and larger towns where abattoirs are available, I believe it is just as well to secure blood from slaughtered animals. Once every two or three weeks a sufficient supply may be obtained in this manner by the citrate-formalin method described later, which enables one to preserve the corpuscles for several weeks and greatly reduce the time and labor involved. When cattle blood is being employed it is usually necessary to secure it from an abattoir and the same method of collection and preservation may be employed.

When however, a sheep maintained for complement-fixation work is bled too frequently or too profusely, an anemia may develop which may materially alter the resistance of the red blood corpuscles even when due precautions are taken to use suspensions containing the average number of erythrocytes per cubic millimeter of suspension. This has occurred repeatedly in my experience and Dr. Hauenstein of Buffalo and others have reported similar experiences to me. In laboratories using an antisheep hemolytic system it is customary to keep one or more sheep for complement-fixation work; a few workers believe that best results are secured by using an immune hemolysin prepared with the blood of the same sheep as used for the hemolytic antigen. But for ten years I have used the blood of sheep secured from an abattoir for hemolytic antigen and for purposes of immunization, with entirely satisfactory results; so far as I can ascertain maintaining a sheep for complement-fixation work is only a convenience and by no means a necessity, provided blood can be conveniently secured from an abattoir at least once a week or even less frequently if the cells are preserved. Lyon[1] has

reported that blood obtained from an abattoir is as well suited for hemolysis work as the blood from a single animal.

Variation in Resistance of Erythrocytes of Sheep to Serum Hemolysis. —One slight advantage of maintaining sheep as a source of blood is the use of corpuscles of uniform resistance to serum hemolysis. Ottenberg and Hopkins[2] have found considerable variation in the resistance of cells from different sheep, although Ottenberg[3] regards the range of variation as exerting but a minor role in the irregularities in the results of the Wassermann test. Brown and I[4] tested the corpuscles of 10 sheep at a time on three different occasions, employing the same complement and hemolysin for each batch of 10 bloods, but found practically no variations in resistance. However, when blood is obtained weekly from an abattoir it has been my experience that once or twice during the year corpuscles of greatly increased resistance may be encountered. The nature of this increased resistance is unknown. Miss Yagle and I have observed that the cells absorb as much antisheep hemolysin as average cells; therefore it is not due to lack of sensitization. But the sensitized cells require more complement for producing hemolysin; why this should be so I am unable to state.

Possibly the breed of sheep may have an influence upon resistance of corpuscles to hemolysis; Dr. Laurence R. Hall informs me that in his experience the corpuscles of the mutton type black nose sheep are almost always more resistant than the cells of the fine wool sheep.

Influence of the Method of Collecting Blood Upon the Erythrocytes.— In collecting blood for the purpose of using the erythrocytes for indicator antigen, three methods are commonly employed, namely, (*a*) bleeding into a suitable vessel and defibrinating with glass beads or rods; (*b*) bleeding into an anticoagulating fluid, and (*c*) breaking up coagula of blood and thereby liberating erythrocytes. The latter method is frequently used for securing human cells from specimens of blood submitted for the Wassermann reaction.

In a study of these methods Brown and I reached the following conclusions:

1. The method of collection has but very slight influence upon the resistance of the erythrocytes, provided the cells are washed one or more time to remove all traces of liberated hemoglobin and serum.

2. Cells secured by defibrination yield the same results as those secured with an anticoagulating fluid.

3. Cells collected in plain sodium-citrate-saline solution yield identical results with those collected in sodium-citrate-gelatin-saline solution.

4. Cells collected by breaking up coagula are apt to require more washing to remove liberated hemoglobin and may be found somewhat more fragile.

5. In collecting small amounts of blood, as human blood or blood from an ear vein of a sheep, it is better and more economical to use an anticoagulating fluid because all of the blood is thereby saved.

Influence of Washing Erythrocytes Upon Their Suitability for the Complement-fixation Reaction.—The original Wassermann test called for three washings of the erythrocytes in physiological saline solution and this practice has been adhered to almost universally. The purposes of washing are to remove all traces of serum and any hemoglobin liberated by mechanical or spontaneous disintegration of red blood corpuscles. It is necessary to remove all traces of serum in order to (1) avoid possible precipitin reactions; (2) to avoid possible anticomplementary activity of the animal's serum if the blood is more than a day old; (3) to avoid anaphylactic reactions if the cells are used for purpose of injecting rabbits at long intervals in the preparation of hemolysin and (4) to avoid erroneous Wassermann reactions, if human corpuscles are being employed, by carrying over syphilitic serum with the cells.

In our experience washing with ordinary 0.85 per cent sodium chloride solution is just as satisfactory as Locke's and Ringer's solutions. Corpuscles washed only once or twice may carry over enough sheep serum to give anticomplementary reactions; they should be washed at least three times with at least 4 volumes of saline solution before being prepared in suspension.

Method of Preparing a Suspension of Washed Red Blood Corpuscles in Relation to the Complement-fixation Reaction.—Considerable influence upon the results of serum hemolysis is exerted by the manner with which the suspension of washed cells is prepared. In some laboratories they are measured with a pipette and suspended in saline solution; in others the volume of washed cells is read off directly in the graduated centrifuge tube in which they were washed; *the results vary according to the method of measuring the cells and especially according to the duration and speed of centrifugalization of the last washing.* For example, the experiments made by Brown and myself showed that suspensions prepared of cells centrifuged for ten minutes or less, contained fewer erythrocytes and required less complement to produce hemolysis, than suspensions prepared of cells centrifuged for thirty minutes. The variation was much greater between five and ten minutes than between ten and thirty minutes, owing to the fact that with high speed the great majority of cells were thrown down within the ten-minute interval. But while great variations in the concentration of corpuscle suspensions showed an influence upon the complement and hemolysin titration, *slight differences in the actual number of erythrocytes present in different suspensions did not produce apparent variation in the results of serum hemolysis.* In our experiments the complement serum was diluted 1 to 20 and used in amounts increasing by 0.1 cc. with a constant

dose of hemolysin; under these conditions it appeared that a difference of at least 100,000,000 erythrocytes per cubic centimeter of suspension was required before a definite influence upon the results of a complement titration became apparent.

Slight variation in the concentration of corpuscle suspension, therefore, exerts but little influence upon the results of the complement-fixation test provided the same suspension is used in the tests as in the preliminary complement and hemolysin titrations. But chances of error are present if one suspension is used for the titrations and a second for the main tests unless both are prepared in the same manner. Furthermore, if the hemolysin is used in a fixed amount depending upon the results of a previous titration, the complement unit will vary not only because of a possible difference in the activity of the complement serum but also on the basis of variation in concentration of the hemolytic antigen. For these reasons it would appear advisable to employ a fixed and uniform method for the preparation of the corpuscle suspension although it is not required to count the erythrocytes per cubic millimeter of suspension or estimate the hemoglobin as recommended by some serologists. This method recommended by me (see Chapter XXXI) measures the corpuscles in a graduated centrifuge tube and has proven very satisfactory.

The Preservation of Sheep Blood. This subject has deservedly attracted considerable attention because a good and satisfactory method for the preservation of blood for complement-fixation work is sometimes necessary in the interests of necessity, economy or convenience. It is now my practice to use formalized blood entirely for conducting the tests and for immunizing rabbits and the results have been entirely satisfactory. By securing and preserving sheep blood once every two or three weeks according to the method described in Chapter XXXI, both labor and expense have been reduced.

Four methods have been tried, although the majority of workers prefer the use of fresh blood: (1) the formalin method of Armand-Delittle and Launoy,[5] who added 1 to 300 to 1 to 200 formalin to washed blood which was then kept at room temperature; (2) a similar method advocated by Bernstein and Kaliski,[6] consisting in adding 0.5 cc. of formalin to 400 cc. of defibrinated blood (approximately 1 to 800 dilution of formalin); (3) the method of Rous and Turner,[7] consisting in collecting blood in Locke's solution containing 1 per cent sodium citrate in the proportion of 1 part of blood to 4 parts of solution, washing three times in Locke's solution containing 0.25 per cent gelatin and preserving in Locke's solution plus 2.8 per cent saccharose and (4) the method of Browning and McKenzie,[8] who state that blood may be kept for some weeks in the ice chest if one-tenth its volume of 1 per cent acriflavin or proflavin in normal saline is added to it.

Bernstein and Kaliski found that defibrinated sheep blood preserved with 1 to 800 formalin proved satisfactory for two months, whereas plain defibrinated blood was generally unfit for use after seven days; also that blood collected in a 1 per cent solution of ammonium oxalate as an anticoagulant, followed by the addition of formalin to 1 to 800, kept as well as defibrinated blood with the same amount of formalin. These investigators found that formalin in dilution as low as 1 to 200 was without effect upon the Wassermann reaction and that formalized cells could be used for the immunization of rabbits in the production of antisheep hemolysin. Experiments with citrated human blood with the addition of formalin to 1 to 400 dilution yielded satisfactory results over a period of four weeks.

Reimann[9] in a study of both methods with sheep blood found that formalized cells (Bernstein-Kaliski) were satisfactory for four weeks and that saccharose-preserved cells (Rous-Turner) were satisfactory from twenty-one to twenty-five days, when kept in sealed ampoules. This investigator compared both methods of preservation against fresh sheep blood in complement and hemolysin titrations and in the conduct of 150 to 200 Wassermann tests. Douglas and Brigger[10] found that sheep cells were well preserved by the Rous-Turner method for at least fifteen days, their resistance gradually decreasing. In their experiments the cells were preserved better at 9° C. than at 20° C.

With the assistance of Dr. Brown and Miss Yagle,[11] I have studied these and other methods with considerable interest and the results may be summarized as follows:

1. The temperature at which blood is kept, regardless of the method of preservation, exerts an important influence. Alternate freezing and thawing results in hemolysis, but blood kept at 2° to 4° C. is better preserved than at 8° to 10° C. and the higher temperatures are increasingly less favorable for preservation.

2. Plain defibrinated blood may be preserved at a temperature under 8° C. for at least ten days. After four days there is an apparent decrease in resistance shown by the lesser amounts of complement required for complete hemolysis but the cells are satisfactory. After ten days spontaneous hemolysis is greater and the blood becomes darker in color and gelatinous in consistency. Defibrinated blood kept at 18° to 20° C. is generally unfit for use after five days.

3. Defibrinated blood or blood collected in an anticoagulant fluid, washed three times with plain saline solution and kept in a mass in centrifuge tubes under the saline solution of the third washing at a low temperature, is usually well preserved for at least seven to ten days; after two weeks the cells are quite fragile, require more than three washings, yield a small complement unit, and in the complement-fixation reaction tend to break down upon standing over night.

4. Blood collected and kept in Locke sodium citrate solution and defibrinated blood kept in plain Locke's solution and in Locke gelatin-sodium citrate solution in the proportion of 1 part blood to 4 parts solution, are generally quite fragile and dark in color in seven days.

5. Blood collected, washed and preserved according to the Rous-Turner method, is found satisfactory for two weeks. After fourteen days the cells become more fragile; in eighteen days the saline solution of the first and second washings is tinged with hemoglobin, the complement unit is reduced and the cells undergo slight spontaneous hemolysis.

6. Formalized cells by the Bernstein-Kaliski method are well preserved for at least fourteen to twenty-one days; after that time they become slightly less resistant but usable up to the twenty-fifth day. After twenty-eight days they are quite fragile, somewhat dark in color, require four or five washings, hemolyze with a small complement unit and undergo considerable spontaneous hemolysis.

7. Formalized cells by the Delittle-Launoy method tend to become dark and slimy within a week and the method was found inferior to that of Bernstein and Kaliski.

8. Preservation with acriflavin was found satisfactory for about two weeks providing the acriflavin was added to the unwashed blood; when added to washed sheep corpuscles, agglutination and hemolysis were sometimes produced. Even four washings may not remove all of the yellow color but what remains does not appear to affect the cells, the complement or the results of titrations and complement-fixation reactions.

9. Sheep blood corpuscles preserved with formalin by the Bernstein and Kaliski method or with acriflavin by the Browning and McKenzie method, were found suitable for the immunization of rabbits for periods up to three weeks and sometimes for an additional one or two weeks.

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CHAPTER XI.

COMPLEMENT SERUM FOR THE COMPLEMENT FIXATION TEST; THE PRESERVATION OF COMPLEMENT.

Following the custom of Bordet and Gengou who employed guinea-pig serum for complement in their original complement-fixation experiments, Wassermann and his associates adopted the serum of this animal for complement in the syphilis reaction, and this practise continues in the majority of laboratories. Whether or not Bordet and Wassermann decided upon this animal because of its general laboratory usefulness and availability or after comparative experiments with the sera of other animals I am unable to state, but such experiments by others have indicated that the serum of the guinea-pig is best suited for complement among the sera of the lower animals and that Bordet's choice was both excellent and fortunate.

That the complement employed in the Wassermann and other complement-fixation tests may vary sufficiently in essential properties to constitute an important source of error and a cause for variation in results with the same serum in different laboratories is generally accepted, and a thorough practical knowledge of the properties insofar as they concern the phenomenon of complement fixation, is required for the conduct of the Wassermann and other complement-fixation tests under acceptable conditions.

REQUIREMENTS OF AN ACCEPTABLE COMPLEMENT SERUM.

Because of our lack of definite information regarding the mechanism of complement-fixation one must accept the total of practical experience of investigators in this field of immunity as our guide and upon this basis it may be stated that an acceptable complement serum should possess certain properties as follows:

1. *A high degree of hemolytic activity for the erythrocytes of the indicator antigen,* that is, prove hemolytic in relatively small doses; even if satisfactory in other respects a complement required in large amounts for complete hemolysis is apt to prove unsatisfactory if we accept the statements of Noguchi and Bronfenbrenner[1] that fixation is not selectively directed toward complement, but that an excess of indifferent serum proteins may result in their fixation by syphilis antibody and antigen and thereby deviate complement from fixation by syphilis antigen and antibody resulting in erroneous reactions tending toward falsely negative results.

2. *A high degree of fixability by the antigen-antibody complex;* upon this property largely depends the delicacy of the reaction and serologists are too prone to overlook the fact that the complements of all animals vary considerably in their fixability. I have never found a complement serum of human beings or guinea-pigs totally devoid of fixability when tested with several different syphilitic sera although they may not be fixed by every syphilitic serum. In other words *a complement may be fixed by one*

serum and antigen and not by another, and it is an error to conduct Wassermann and other complement-fixation tests with the complement serum of one animal; to do so constitutes a frequent cause for varying results when several serologists test the same serum, and particularly if this serum yields a weakly positive reaction.

3. *It should not be too susceptible to non-specific fixation*, that is, not too sensitive to the so-called anticomplementary effect of serum alone and antigen alone; this is a factor of much importance and one generally overlooked in reference to complement.

4. *It should not contain natural amboceptors or complement-fixing antibodies* for the antigens employed in the Wassermann test or for the usual bacterial antigens like those of the *gonococcus*; *B. tuberculosis*, *B. typhosus*, *B. mallei*, etc., employed in complement-fixation tests.

5. *It should be adaptable to the hemolytic system being employed*, that is, be free, or largely so, of agglutinins for the corpuscles of the indicator antigen and also of the hemolysin for these cells; the agglutinin is of more importance, and when present, as is likely to be the case in human serum, frequently interferes with hemolysis particularly in an antihuman hemolytic system.

6. *It should be available;* which refers to the readiness, ease and economy with which this constituent of the complement-fixation test may be supplied.

HEMOLYTIC ACTIVITY OF COMPLEMENT.

A comparative study of the complement of human beings, guinea-pigs, white rats, rabbits, sheep, hogs and cattle by Matsunami, Trist and myself[2] has shown that guinea-pig serum is most hemolytic for sensitized sheep corpuscles; likewise for sensitized cattle and human corpuscles. Noguchi and Bronfenbrenner[3] in a prior study of the complements of the guinea-pig, cat, dog, rabbit, rat, hog, ox, sheep and chicken reached the same conclusion relative to the antihuman system; Yui, Tyau and myself[4] in a study of the complements of the ordinary (*mus norvegicus var. albinus*) and wild sewer rats (*mus norvegicus*) found that they possessed less hemolytic activity for sensitized sheep and human cells as compared with guinea-pig serum, but that they were quite fixable in the Wassermann test and could be employed in an antihuman system. Table 6 shows how these complements compare with guinea-pig serum in hemolytic activity:

TABLE 6.

Complement.	Relative hemolytic activity.*		Absolute hemolytic activity.†	
	Antisheep system.	Antihuman system.	Antisheep system.	Antihuman system.
Human	6–7 x weaker	5 x weaker	10 x weaker	6–7 x weaker
Rat	3 x weaker	About equal	3–4 x weaker	½ x weaker
Rabbit	10 x weaker	2 x weaker	10 x weaker	2–3 x weaker
Hog	3–4 x weaker	About equal	5–6 x weaker	½ x weaker
Sheep	70 x weaker	3 x weaker	70 x weaker	3 x weaker
Ox	70 x weaker	About equal	70 x weaker	2 x weaker

* Before removal of natural hemolysins and agglutinins for sheep and human cells.
† After removal of natural hemolysins and agglutinins for sheep and human cells.

In general terms guinea-pig complement is from six to seven times more hemolytic for sensitized sheep corpuscles than human complement and is certainly the complement of choice in the antisheep, antiox and antihuman systems. *The hemolytic activity of the complements of different guinea-pigs varies however, and uniformity is secured only by using a mixture of sera.*

The sera of some guinea-pigs, however, are notably lacking in complement; according to Moore[5] this property is inheritable and is not due to the presence of anything interfering with the action of hemolysin. Coca[6] states that in such complement both the mid-piece and end-piece are present but that the so-called "third piece" is lacking, the addition of inactive guinea-pig or human serum containing this third component to the deficient complement increasing its hemolytic activity about three times. Ecker[7] found that by greatly increasing the amount of hemolysin these deficient complements could be made to produce hemolysis but in my experience Wassermann and other complement-fixation tests conducted with them are almost always unsatisfactory and especially because they are hypersensitive to the anticomplementary effects of antigen alone and serum alone and thereby tend to yield non-specific reactions.

FIXABILITY OF COMPLEMENT.

Of greater importance from the standpoint of the Wassermann and other complement-fixation tests is the fixability of complement by antigens and antibodies inasmuch as hemolytic activity alone is no criterion. In a study by Matsunami, Trist and myself of the sera of human beings, guinea-pigs, rats, rabbits, sheep, hogs and cattle it was found that human complements were most susceptible to fixation by syphilis antigen and antibody with guinea-pig running a close second. Rat and rabbit complements usually gave good fixation; hog complement was fair while that of sheep and cattle was very poor. Additional investigations in tuberculosis, typhoid, glanders and gonococcus complement-fixation tests with human and guinea-pig complements have yielded similar results, that is, human complement was found slightly more fixable. This is particularly true in those methods employing the native complement of each patient's serum as shown in a study of Flick and myself[8] of the methods of Tschernogubow, Emery, Thompson, Ronchese, Noguchi, Bronfenbrenner and Schlesinger, Stern, Foix, Hecht, Flemming, Gradwohl, Bartlett and O'Shansky. But in tests of these kinds there is a greater tendency to non-specific Wassermann reactions and especially if crude alcoholic tissue extracts are employed as antigens. Furthermore the native complement of a patient's serum is sometimes deficient in fixability and may thereby yield a falsely negative reaction. In tests like those of Butler and Landon, Health, Myer,

Cress and Noguchi employing normal human serum as a substitute for guinea-pig serum as complement Flick and myself found human complement much less satisfactory and in a special study of Noguchi's test[9] in syphilis and gonococcus infections I found[10] human complement decidedly unsatisfactory by reason of the occurrence of a high percentage of anticomplementary or indefinite reactions.

Guinea-pig serum complement is to be preferred but owing to variation in fixability by all antigens and antibodies a mixture is essential for best results and by the use of superior antigen along with ice-box primary incubation of at least eighteen hours it is possible to render the reactions as sensitive as those obtained by employing the complement of each patient's serum without as much danger of non-specific reactions.

SUSCEPTIBILITY OF COMPLEMENT TO NON SPECIFIC COMPLEMENT-FIXATION.

Of considerable interest and importance in this connection is a further consideration of this important question of the susceptibility of the complements of various animals to non-specific fixation by antigen alone and patient's serum alone—well known as the anticomplementary action of antigen and serum. For example, sheep and ox complements usually yield strongly positive reactions, but both are very susceptible to antigen alone and serum alone and but slightly to specific fixation in mixtures of antigen and serum. As will be described later, all complement sera become more sensitive to non-specific fixation and anticomplementary influences according to the method of preparation and age of the serum, but experiments with the complements of the animals mentioned above and collected in a uniform manner have shown that *guinea-pig complement is less susceptible to non-specific fixation and anticomplementary influences than the complement of any other animal: human complement is more susceptible* and for this reason is more likely to yield unsatisfactory serum and antigen controls, in that interference with hemolysis is of frequent occurrence. This is true not only in the Wassermann test but likewise in bacterial complement-fixation tests and it may account in part for the impression that human complement is more susceptible to *specific* fixation, On the whole therefore, while guinea-pig complement may be slightly less fixable it is certainly much less susceptible to non-specific or anticomplementary effects and is without doubt the complement of choice. Furthermore and very fortunately, *guinea-pig serum (both unheated and heated) does not give the non-specific complement-fixation reactions with Wassermann and various bacterial antigens so commonly exhibited by normal rabbit, dog and mule sera.*

COMPLEMENT-FIXING ANTIBODIES IN COMPLEMENT SERUM.

To the best of my knowledge the complement sera of guinea-pigs have never been found to contain the syphilitic antibody concerned in the Wassermann reaction; they cannot be infected with *Spirochæta pallida* nor do they suffer with other spirochetic diseases apt to produce this substance. But the subject is of more importance in relation to some of the bacterial complement-fixation tests and particularly tuberculosis because of the possibility of the blood of these animals sometimes containing natural complement-fixing antibodies for bacterial antigens. Pritchard and Roderick[11] and Upham and Blaivas[12] have drawn attention to this possibility in relation to the tuberculosis test but otherwise the subject has not commanded much attention.

I have tested the sera of a large number of adult healthy pigs of both sexes for complement-fixing antibody with approximately 20 different antigens of tubercle, typhoid and glanders bacilli and gonococci; also with a few antigens of diphtheria bacilli, pneumococci, meningococci and a ringworm fungus (*Trichophyton rosaceum*). The serum of an occasional animal appeared to yield a weak reaction with some of the tubercle antigens but in no instance were we able to find tuberculous lesions. Negative reactions were observed with all other antigens.

I believe, therefore, that if the sera of healthy, unused, full-grown guinea-pigs are used there is almost no danger of positive reactions due to the presence of antibody in the complement; *furthermore, since the complement is titrated in the presence of the antigen in my bacterial complement-fixation technique this possibility is always and automatically taken care of because if a trace of antibody were present the unit of complement would be just so much greater on account of the fixation of a portion by antigen and antibody.*

Needless to state the use of human serum as a substitute for guinea-pig complement as in Noguchi's hemohemolytic system is far more likely to introduce error due not only to the presence of unsuspected clinically concealed syphilis in human individuals but likewise to the possible presence of antibodies for the gonococcus and other bacterial antigens.

HEMAGGLUTININS AND HEMOLYSINS IN COMPLEMENT SERUM.

As stated in the preceding chapter, guinea-pig complement is practically free of natural agglutinins for the erythrocytes of human beings, sheep, cattle and chickens; about 40 per cent, however, contain natural hemolysins for sheep, cattle and chicken corpuscles. In my experience hemagglutination is never a disturbing factor in the antisheep system employing guinea-pig complement and the

same may be stated for the antiox system when sufficiently powerful hemolysin is employed. Agglutination, however, may be very disturbing in the antihuman and antichicken systems, not so much because of agglutinins in the complement or patient's sera but because of their presence in the immune hemolysin sera.

Even though guinea-pig complement may contain natural hemolysin for the erythrocytes being used, it is not a disturbing factor in my test because the immune hemolysin is routinely titrated against a fixed amount of the complement which thereby makes the proper quantitative adjustment of the hemolytic system regardless whether it is the antisheep, antiox or antihuman.

VARIATION IN THE HEMOLYTIC ACTIVITY AND FIXABILITY OF COMPLEMENT.

Not only may the complement sera of animals of different species show marked variation in lytic activity and fixability, but what is more important, the complements of animals of the same species may show differences in lytic activity and particularly in fixability by syphilis serum and antigen as shown by Browning and McKenzie[13] and confirmed by the investigations of Clark,[14] Noguchi and Bronfenbrenner,[15] Ottenberg,[16] Detweiler,[17] Ruediger[18] and others; these investigators have found quite uniformly that guinea-pig complement serum is apt to vary more in fixability than in lytic activity and that *in order to overcome this source of error a mixture of sera from several pigs should be used.*

These warnings have not received the attention they deserve and it is absolutely necessary to control the factor of fixability of complement for acceptable work with the Wassermann and other complement-fixation tests. It is a widespread custom and particularly in smaller laboratories, to depend upon the complement serum of one animal. While I have never found a guinea-pig complement devoid of fixability when tested with the sera of several syphilitic individuals, yet *a complement may not be fixed by one or more particular sera in a series of tests, that is, a complement may be fixed by one syphilitic serum and not by another and thereby prove misleading. The same is true in bacterial complement-fixation tests.* I have found the same true of human complement and this fact constitutes one source of error in methods employing the complement of each patient's own serum as those of Hecht, Gradwohl, Tschernogubow, Thompson, Noguchi and others as already stated.

The results of the experiments of Matsunami, Trist and myself have shown that in an antihuman system about 50 per cent of human complements will vary to some extent *in hemolytic activity* from a uniform unit and about 30 per cent of guinea-pig complements will show some variation. In an antisheep system about 65 per cent

human and 30 per cent guinea-pig complements show some variation. These results are presented in Table 7; the variations were very slight in the majority of instances and only demonstrable by strict attention to details in the titrations:

TABLE 7.

Complements.	Percentage of complements showing variation in hemolytic activity.	
	In antisheep system.	In antihuman system.
Human	65 per cent	50 per cent
Guinea-pig	30 per cent	30 per cent
Rat	50 per cent	50 per cent
Rabbit	60 per cent	60 per cent
Sheep	Not determined	65 per cent
Hog	50 per cent	65 per cent
Ox	Not determined	50 per cent

Our tests for *variation in fixability* were conducted after the method of Browning and McKenzie as it impressed us as being the only accurate means for determining the fixability of a complement.

The general results have shown: (1) that both guinea-pig and human complements vary in fixability in the Wassermann and other complement-fixation reactions and (2) that human complements are more apt to show this variation in fixability than guinea-pig complement. According to our experiments about 50 per cent of human and about 30 per cent of guinea-pig complements show variation in fixability. Doubtless this is a potent factor for error and an explanation in part of the variable results reported by different laboratories testing the same serum and particularly if the serum of one pig is relied upon for furnishing complement.

SINGLE AND MULTIPLE COMPLEMENTS IN THE CONDUCT OF COMPLEMENT-FIXATION TESTS.

By reason of this known variation in the fixability of guinea-pig complement many serologists, probably a majority of those working in large laboratories, have employed a mixture of complement sera in conducting complement-fixation tests. In smaller laboratories conducting relatively few tests per week single complements are generally employed.

While the advisability of using multiple complements is based upon the results of titrations of guinea-pig complements, actual comparative Wassermann tests employing single and mixed complements with a large number of selected sera do not appear to have been reported upon and Matsunami, Trist and myself have considered the question of sufficient importance to devote considerable time to it. Accordingly 126 selected sera were subjected to the Wassermann test employing three antigens, namely cholesterinized

alcoholic extract of human heart, alcoholic extract of syphilitic liver and acetone resoluble lipoids and the results have proved of great interest and brought out several important facts as follows:

(*a*) *A mixture of several complements yielded a higher percentage of true positive reactions than single complements;* the results varied with the antigens as was expected, the cholesterinized extract yielding the largest number of positive reactions and the alcoholic extract of syphilitic liver the least.

(*b*) *A mixture of several complements was found not as apt to yield falsely positive reactions as a single complement;* of the 40 sera of normal healthy persons tested the cholesterinized extract yielded 3 and the alcoholic extract of syphilitic liver 1 weakly positive reaction in the tests conducted with one complement whereas with the mixture of complements all tests were clearly negative. In other words, *a single complement is more susceptible to non-specific fixation or the anticomplementary action of antigen than a mixture of complements* and this may have a bearing upon the unfavorable reports of several serologists in reference to cholesterinized extracts yielding falsely positive results.

(*c*) *A mixture of complements was not as susceptible to the anticomplementary action of serum as a single complement;* of the 126 sera studied, 26 were anticomplementary in tests employing one complement, while only 6 of these were anticomplementary with the mixture of complements. In no instance was a serum anticomplementary with the mixture of complements alone; in other words *some complements are more susceptible than others to anticomplementary influences and in a mixture of several this tendency is reduced to a minimum.*

THE INFLUENCE OF THE METHOD OF PREPARATION UPON THE HEMOLYTIC ACTIVITY AND FIXABILITY OF GUINEA-PIG COMPLEMENT.

Various methods are employed in different laboratories for the collection and preparation of guinea-pig complement serum. For example, in some laboratories the animals are bled the day before and the serum allowed to separate in the laboratory or in the refrigerator; in others the animals are bled on the day the serum is required, one or several hours before being used. A question of practical importance is whether or not the lytic activity and fixability of a serum is influenced by the method of collection.

Browning and McKenzie found that freshly drawn serum is hypersensitive to the anticomplementary activity of antigen alone and patient's serum alone and advocate keeping a serum on ice for eighteen to twenty-four hours before use; Gurd found that guinea-pig and human complement sera gradually increased in activity

if kept for an hour in the incubator and then in the refrigerator, when they would remain constant for twenty-four to forty-eight hours followed by deterioration. Noguchi and Bronfenbrenner found that guinea-pig sera left in contact with the clot for forty-six hours were usually more active than after twenty-four hours (a few became weaker), and that to insure greater uniformity it was preferable to let sera remain in the clots for at least twenty-four hours before use. These investigators also found that variation in fixability was more marked than variation in lytic activity under the condition of their experiments.

Of primary importance in this connection is whether or not complements exist preformed in plasma; Gengou[19] originally claimed that they did not but developed only upon the production of serum after coagulation had taken place. Considerable work has since been conducted on this problem with conflicting results; recently Watanabe[20] working in my laboratories, perfected a method for the collection of plasma which proved generally satisfactory and in a series of very carefully executed experiments demonstrated the presence of hemolytic complement in the plasma of persons and the lower animals in amounts closely similar to the corresponding sera and likewise the presence of such antibodies as hemolysins, agglutinins and the antibody concerned in the Wassermann reaction.

My experiments upon the influence of method of preparation of complement were conducted with guinea-pig and human complements and consisted in collecting and dividing each specimen into five or six equal parts in small sterile centrifuge tubes. *One* part was broken up at once with a glass rod and centrifuged for the serum. A *second* part was placed in an incubator at 38° C. for one hour, broken up and centrifuged. A *third* part was placed in the incubator for an hour and then in a refrigerator for twenty-four hours; if the serum had not separated thoroughly the coagulum of blood was gently broken up and centrifuged. A *fourth* part was allowed to separate at room temperature requiring generally five or six hours. A *fifth* part was placed in the refrigerator for twenty hours and a *sixth* for forty-eight hours.

Each specimen was then titrated for hemolytic activity with the same dose of hemolysin and same dose of the same corpuscle suspension; in this manner an attempt was made to make the tests rigidly comparative for the five or six parts of each specimen. In the fixation tests the same syphilitic serum and same antigen were employed. The results have been summarized as follows:

(*a*) With the methods employed variations in hemolytic activity and fixability were slight and inconstant.

(*b*) As a general rule, complement secured by defibrinating the blood immediately after collection and centrifuging for the serum was slightly inferior in hemolytic activity and fixability and occasionally hypersensitive to the anticomplementary influence of antigen alone and serum alone, as stated by Browning and McKenzie.

(*c*) Complement secured by placing the blood in the incubator for an hour before breaking up and centrifuging the clot generally acquired an increase of lytic activity and fixability as stated by Gurd; for example a

guinea-pig complement proving completely lytic in 0.03 cc. secured immediately after bleeding would be lytic in 0.025 cc. when secured from a portion of the same blood placed in the incubator for an hour before centrifuging. With human complement the same differences were frequently noted and usually in a slightly greater degree.

(*d*) Complement serum which separated from clots at room temperature over a period of six to twelve hours generally possessed a degree of lytic activity and fixability as good as yielded by any method.

(*e*) Blood placed in the refrigerator immediately after collection and the temperature rapidly reduced to 2° C. and kept for eighteen to twenty-four hours generally yielded a complement serum of reduced lytic activity although fixability was not influenced. After forty-eight hours the lytic activity was the same or slightly decreased and fixability apparently increased due to increased sensitiveness of the complement to antigen alone and serum alone which yielded reaction suggestive of possible increased fixability of complement but not an actual or absolute increase.

THE INFLUENCE OF AGE, SEX, HEALTH, DIET, REPEATED BLEEDINGS AND ANESTHESIA OF GUINEA-PIGS UPON THE PROPERTIES OF COMPLEMENT.

Matsunami, Trist and I have been unable to find reports in literature bearing upon these subjects beyond isolated statements of several authors that complement derived from sickly animals or from persons suffering from febrile diseases is apt to be poor in hemolytic activity. The question, however, of the possible influence of age, sex (pregnancy), general health, feeding, repeated bleedings and anesthesia of guinea-pigs in relation to the properties of their complement insofar as complement fixation is concerned, has been considered worthy of special study.

(*a*) **Influence of Age.**—The sera of 12 guinea-pigs weighing from 200 to 280 gms. compared with the sera of 12 weighing from 400 to 580 gms. showed no variation in hemolytic activity and fixability beyond those variations possible and to be expected due to individual differences. These animals were taken from the same pens, received the same food and were bled at the same time and the sera collected in the same manner, that is, each specimen was placed in the incubator for one hour followed by breaking up of the clots and centrifuging for the sera which were tested at once for hemolytic activity and fixability. But sera from *younger pigs were frequently more sensitive to the anticomplementary activity of antigen alone and serum alone; large heavy animals are always most likely to yield complements of remarkably uniform lytic activity and fixability and should always be preferred.*

(*b*) **Influence of Sex (Pregnancy).**—Sera from bloods secured from the hearts of 16 pregnant pigs weighing over 450 gms. compared with sera collected at the same time and in the same manner from males of corresponding weight and living under identical conditions, showed no constant changes in hemolytic activity and fixability.

Occasionally sera collected near term and within twenty-four hours after delivery were poorer in hemolytic activity and somewhat hypersensitive to the anticomplementary influence of antigen alone and serum alone.

(*c*) **Influence of General Health.**—Experiments with groups of pigs weighing from 200 to 300 gms. suffering with generalized tuberculosis of over fourteen days' duration or within one to three weeks after injection with diphtheria toxin or cultures of the diphtheria bacillus as the survivors of tests for toxicity and virulence, have shown with fair uniformity that the complements are below the average in hemolytic activity and quite hypersensitive to the anticomplementary action of antigen alone and serum alone. *Any guinea-pig that is thin and wasted from these or other causes is so apt to yield an unsatisfactory complement serum that it should not be used.* On the other hand, pigs surviving injections of diphtheria and tetanus toxins and antitoxins given some months previously, in good general health and of approximately normal weight are entirely likely to yield acceptable complement sera; in our experience however, a large number of these animals remain undersized and undernourished and frequently yield unsatisfactory complement sera.

(*d*) **Influence of Diet.**—The influence of diet upon the properties of complement is difficult for us to evaluate in the absence of systematized and scientifically regulated diet experiments; our animals were given a mixed diet of uncooked vegetables and grains (principally oats) according to the season of the year and among those animals of good health and normal weight we have never noted any constant changes in the properties of complement except on two occasions when pigs fed on cabbage for several weeks, with occasional meals of carrots or beets, yielded complements poor in hemolytic activity and quite sensitive to the anticomplementary influences of serum and antigen. This subject is worthy of systematized study because of its general import; *insofar as complement for Wassermann tests is concerned, mixed diet maintaining the animals in good health and of normal weight is all that is required.* Ruediger[21] states that a mixture of raw vegetables gives the best results and that the fixability of guinea-pig complement is apt to be better in winter than in summer which may be due to the large quantity of grass fed in summer.

Of equal interest and importance in this connection is the influence of feeding upon the properties of complement. We have removed 2 cc. of blood from the jugular veins or hearts of a series of large animals after starvation for twenty-four hours and again after a meal of vegetables and oats and compared the two sera of each animal for hemolytic activity and fixability with the general result that the sera of the first bleeding, that is, from starved or rather hungry animals, was superior in lytic activity and fixability to sera

removed within an hour after feeding and frequently chylous in appearance. For this reason *it appears advisable to regulate the time of feeding in such manner that animals are bled for Wassermann tests at least six to twelve hours after feeding.*

(*e*) **Influences of Repeated Bleedings.**—Now that it is a growing custom in laboratories to bleed guinea-pigs from the heart or jugular veins in order to secure small quantities of blood from several animals without killing them, to meet the requirements of using a mixture of complement sera and for purposes of economy, a question of importance concerns the possible influence of repeated bleedings upon the properties of complement. The experiments which we have conducted have shown remarkably little influence on the lytic activity and fixability of complements from pigs bled profusely at intervals of one week (about 15 cc. of blood from 500 to 600 gm. animals) or of similar bleedings at intervals of ten days to two weeks. In 4 animals surviving eight large bleedings at intervals of five days an anemia was produced and these sera were finally distinctly below the average in lytic activity although comparatively unchanged in fixability.

Insofar as we can judge, *healthy adult guinea-pigs may be bled once every two or three weeks in the proportion of 1 cc. of blood per 100 gms. of body weight without deleterious influence upon the qualities of their complements.*

(*f*) **Influence of Anesthesia.**—The brief anesthesia (five to ten minutes) with ether required for bleeding a guinea-pig does not appear to influence the properties of the complement; blood removed from 12 adult pigs before and after a fifteen-minute period of ether anesthesia yielded complements of closely similar hemolytic activity and fixability.

PRESERVATION OF COMPLEMENT.

Since the complements of individual guinea-pigs tend to vary in fixability and a mixture of complements having proved more reliable for the Wassermann reaction than single complements, a satisfactory and practical method for the preservation of complement becomes a matter of considerable economic importance and convenience, particularly in laboratories conducting a small number of tests at one time and too few to require all the mixed complement from two or more exsanguinated pigs.

Probably the best method for securing a mixture of complements without undue waste and expense consists in keeping on hand a stock of large healthy animals and bleeding them in rotation from the heart or external jugular vein as complement is required; for various reasons, however, this method may not be practical for certain laboratories, whereas a reliable method of preserving com-

plement may enable the serologist to exsanguinate at least two or three animals and utilize the mixed complements without waste over a certain period of time.

The oldest method for the preservation of complement is that by freezing as mentioned by Morgenroth, who found that serum kept at —10° to —15° C. preserves the various constituents for a long time; Penecke[22] has also stated that complement may be preserved by this means for four to five weeks. Weston[23] reports that serum at —15° C. showed no appreciable deterioration in hemolytic activity after a period of three months and one of three specimens was preserved for five months. Stern[34] has preserved complement in this manner, but has stated that deterioration in fixability by mixtures of syphilitic serum and organic extracts develops prior to changes in hemolytic activity. More recently Moledesky[25] has advocated the preservation of complement serum frozen solid in test-tubes packed in a mixture of ice and salt in a thermos bottle and states that the serum keeps indefinitely under these conditions.

For several years various salts have been used for the preservation of complement serum and particularly sodium chloride in from 8 to 37 per cent; I have used 17 per cent sodium chloride (0.17 gms. to each cubic centimeter of serum) with considerable success when the serum was kept at a low temperature. By diluting each cubic centimeter of serum with 19 cc. distilled water a 1 to 20 dilution in isotonic salt solution was obtained. Austin,[26] Thompson,[27] Ottenberg,[28] and Neill[29] have also used sodium chloride with success. Rhamy[30] has employed sodium acetate and Ruediger[31] has used glycerin.

Grigorowitsch[32] and Silber[33] have added 5 per cent magnesium sulphate to serum followed by drying and apparently with successful results; Noguchi[34] has dried guinea-pig complement in filter paper as a means of preservation and to simplify the use of this constituent in his test, but found that deterioration occurred rather rapidly. Hartly, Eagleton and Okell[35] have dried complement rapidly *in vacuo* over sulphuric acid and Browning and Dunlop[36] found their product a valuable source of complement for use in the Wassermann test.

Matsunami and myself[37] have conducted a large number of experiments with guinea-pig and human complements preserved with these different methods and in each experiment the preserved complement was tested fresh and at varying intervals for the following three values: (1) for hemolytic activity in an antisheep system; (2) for fixability in mixtures of syphilitic sera and various organ extracts (antigens) and (3) for susceptibility to non-specific fixation by serum and organ extracts alone, better known as the anticomplementary effects shown by the serum and the extract or antigen.

In the majority of published accounts of such experiments it

would appear that hemolytic activity alone was accepted as the criterion of the quality of the preserved serum, whereas in our experiments *we have found that a slight decrease in specific fixability alone or associated with an increased susceptibility to non-specific fixation are the earliest changes of deterioration and that hemolytic activity may be preserved for longer periods in an otherwise unsatisfactory serum.* Hemolytic activity alone therefore is not a safe criterion for preserved complement, a fact that has been emphasized by Stillians.[38]

In my experience, complement is best preserved with sodium chloride and kept at or near the freezing-point. In most laboratories a temperature of −10° to −15° C. cannot be maintained routinely but temperatures of 0° to 4° C. are readily obtained. Lyon and Trager[39] have added 25 per cent sodium chloride and have preserved the serum at 4° to 5° C. on ice for three to four weeks; at room temperature deterioration occurred within ten days.

Miss Richter, Miss Yagle and I have observed similar results and in our experience guinea-pig complement can be preserved satisfactorily for my test for at least four to six weeks by adding 0.25 gms. of chemically pure sodium chloride to each cubic centimeter of serum (25 per cent) and keeping at 0° to 4° C. which is easily possible in an electric refrigerator or by wrapping the bottle or ampoules in a towel on a cake of ice. For use the mixture is shaken and 1 cc. diluted with 29 cc. of distilled or tap water which gives an isotonic (0.833 per cent) 1 to 30 dilution as employed in my method. Unfortunately serum preserved in this manner does not keep well at room temperature (deterioration in a few days) and for this reason the method is not applicable for the distribution of complement by a central laboratory over a wide area because of possible deterioration in the mails.

My experience with dried complement has not been as satisfactory although very encouraging. Some lots prepared by Mr. H. G. Dunham by drying at low temperatures have maintained a high degree of hemolytic activity and fixability for several months but at this writing a uniformly satisfactory method has not yet been worked out. But I believe that the problem will be ultimately solved and efforts along this line are highly desirable because it would enable one to use a highly polyvalent serum with uniform hemolytic activity and fixability at an economic saving.

In the meantime preservation of guinea-pig complement with 25 per cent sodium chloride at 0° to 4° C. can be recommended for use over four to six weeks of time and while a mixture of fresh sera is always to be preferred yet I believe that in small laboratories *it is much better to use the preserved mixed serum of three or more guinea-pigs than to conduct complement-fixation tests with the fresh serum of a single animal.* But even though a serum preserved in this manner may be satisfactorily hemolytic for longer than six weeks my advice

is not to use it after four to six weeks because of a tendency to lose in fixability by Wassermann and bacterial antigens and antibodies after this time.

ARTIFICIAL COMPLEMENT.

Many attempts have been made to prepare artificial complement but without success. Von Lieberman has described a method consisting of heating 1 cc. of normal rabbit serum at 55° C. for thirty minutes, diluting with 9 cc. of saline solution and adding drop by drop 6 cc. of a freshly prepared mixture consisting of 5 cc. of 0.1 per cent sodium oleate in methyl alcohol and 1 cc. of 0.1 per cent calcium chloride in methyl alcohol. Miss Richter and I have observed that 0.6 to 0.8 cc. of this preparation is alone hemolytic for 0.5 cc. of a 2 per cent suspension of washed sheep corpuscles and that the addition of antisheep hemolysin does not materially increase its hemolytic activity; in other words it is without complementary activity.

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CHAPTER XII.

HEMOLYSIN FOR THE COMPLEMENT-FIXATION TEST.

Many methods have been proposed for the preparation of antisheep, antihuman and other hemolysins for the Wassermann test; indeed it would appear that the majority of experienced serologists have adopted a method more or less individual in which they have learned to place much confidence. As a matter of fact antisheep and anticattle hemolysins, and especially the former, are easily prepared by any one of several methods; the preparation of antihuman hemolysin is much more difficult and no method has yet been discovered for producing it as powerful as those usually obtained for sheep and cattle corpuscles.

PREPARATION OF CORPUSCLES.

Hemolysins may be produced by injecting whole blood but it is much better to employ erythrocytes washed free of serum in order to avoid the production of precipitins. Furthermore, animals withstand injections of washed blood better than whole blood and especially by intravenous injection, partly because the serum of whole blood may contain agglutinin for the corpuscles of the immunized animal.

Sheep blood may be obtained from a vein of the living animal in sodium citrate solution or by defibrination but abattoir blood (plain or formalinized) is just as satisfactory. The same applies to cattle blood. Human blood may be obtained from the vein of a volunteer or by adding saline solution to clots, breaking them up with a glass rod and filtering through cotton or wool to remove fibrin and clots.

The corpuscles should be washed at least three times by centrifuging with 3 or 4 volumes of sterile saline solution each time and prepared in a suspension after the last washing.

Ordinary care against gross bacterial contamination is required. Owing to high natural immunity rabbits can withstand the intravenous or intraperitoneal injection of surprisingly large numbers of staphylococci and *B. subtilis* (the usual organisms of contamination) but heavy contamination may produce abscesses with greatly reduced hemolysin production or early death.

There is no particular advantage in injecting sensitized corpuscles in the preparation of antisheep and anticattle hemolysins. In 1900 von Dungern[1] thought that the injection of sensitized cor-

puscles produced little or no hemolysin and Iijima[2] has more recently made a similar statement. But Vedder[3] has shown that in the production of antihuman hemolysin it may be advantageous to sensitize the washed corpuscles for twelve hours with antihuman serum heated at 78 to 80° C. for one hour to destroy agglutinins, as a means for preventing their agglutination when injected intravenously into rabbits and thereby possibly reducing the mortality among the animals. Miss Rule and myself[4] found that sensitized sheep and human corpuscles produced hemolysins in rabbits, sometimes a little more promptly than plain cells, but the end-results were practically identical with those following immunization with the latter and the extra work and use of immune serum are not unjustified.

Some investigators have stated that the injection of hemolyzed corpuscles gave better results than suspensions of whole cells, but this has not been my experience in the preparation of antisheep and antihuman hemolysins. Vedder[5] has advocated injections of stroma of human erythrocytes; Miss Rule and I found that his method produced as much antihuman hemolysin in rabbits as injections of plain corpuscles with somewhat less of the objectionable agglutinin and with a slightly lower death-rate among the animals. But on the whole no better way has yet been found for the production of hemolysins than injections of suspensions of thoroughly washed corpuscles.

SELECTION OF ANIMAL.

Large healthy *white* rabbits have been found well adapted for the production of antisheep and anticattle hemolysins. Their sera usually contains some natural hemolysins for these corpuscles which I believe probably accounts for the readiness with which they yield immune hemolysins. They should be in good health and previously unused; "seconds" frequently yield low-grade hemolysins even though they appear in good health. During immunization they should receive plenty of food, water and exercise (preferably out-doors).

Noguchi and Bronfenbrenner have found rabbits best adapted for the production of antihuman hemolysin. Sanford has recommended the use of small dogs of the fox terrier type. Since the sera of calves and adult cattle usually contain natural antihuman hemolysin it is possible that they would produce the immune hemolysin more readily and in larger amounts than either the rabbit or dog.

ROUTE OF INJECTION AND TOXICITY OF ERYTHROCYTES.

Immunization of rabbits is generally conducted by intravenous or intraperitoneal injections of suspensions of washed erythrocytes.

With the former, hemolysin production occurs somewhat more quickly, requires fewer injections and smaller doses; it is certainly the method of choice for the production of antisheep and anticattle hemolysins.

Rabbits withstand intravenous injections of washed sheep or cattle corpuscles quite well. With proper care they do not lose in weight and the death-rate is very low; intraperitoneal injections are much more likely to give a higher death-rate because of peritonitis.

Washed human corpuscles are much more toxic for rabbits by either route of administration. Most animals are apt to lose in weight and almost 50 per cent succumb before they have produced sufficient hemolysin. Even thoroughly washed corpuscles are apt to produce convulsions and death after two or three injections; these reactions do not appear to be anaphylactic but rather anaphylactoid due to intravascular agglutination. In addition however, human hemoglobin and other dissolved constituents appear much more toxic for rabbits than the products of sheep corpuscles and why hemolysin production to human corpuscles is so much less cannot be stated; either antibody production is suppressed or the antibody-producing tissues are but poorly responsive to the antigenic activity of human erythrocytes. The same is true of chicken and guinea-pig corpuscles for the rabbit.

METHODS FOR THE PRODUCTION OF HEMOLYSINS.

Miss Rule and I found after a careful comparative study of production of antisheep hemolysin in rabbits that almost any method was apt to be satisfactory; possibly one of the best consists in the intravenous injection of 5 cc. of a 10 per cent suspension every three or four days for 5 injections. This is my routine method and is essentially the same as advocated by Kilduffe[6] except that he injects 10 cc. instead of 5 cc. It is also quite useful for the production of anticattle hemolysin.

Another good method is the daily injection of 1 cc. of a 10 per cent suspension (equivalent to 0.1 cc. of packed cells) for 21 to 28 injections as advocated by Coca.[7] Thompson[8] found this method best for the production of antihuman hemolysin and this has been likewise the experience of Miss Rule and myself in a comparative study of six methods. One must be very careful of the ear veins because of the large number of injections required but the death-rate is lower than by other methods and hemolysin production usually superior; it is now my routine method for the production of antihuman and antichicken hemolysins.

Exceptionally good results are sometimes observed by the method brought to my attention by Dr. C. W. Curchin, consisting of giving

white rabbits an intravenous injection of 10 cc. of a 1 per cent suspension of washed sheep cells twice a week for 5 injections, bleeding one week after the last. By this method it is not unusual to secure sera with a titer of 0.5 cc. of 1 to 30,000 to 1 to 40,000 when titrated by my method.

One week after the last injection by either method a small amount of blood is obtained from an ear vein and the serum titrated for hemolysin. If sufficiently good (the unit of antisheep hemolysin should be at least 0.5 cc. of 1 to 4000 or higher) the rabbit may be bled; if too low more injections should be given although some rabbits are poor hemolysin producers and must be discarded. It is always well to immunize at least 4 to 6 rabbits at a time because some will yield hemolysins much higher than others and a mixture of the sera of all together will usually give a satisfactory serum. Not infrequently the titer after bleeding to death is lower than the preliminary titer.

No special precautions are necessary in bleeding the animals. It is unnecessary to take elaborate precautions against bacterial contamination or to expose the carotid arteries, as so frequently advised, when the serum is preserved with an equal part of glycerin as advocated by Clock and Beard.[9] It is sufficient to etherize or stun the animal by a blow at the base of the skull, sever the great vessels on both sides of the neck avoiding the esophagus although the trachea may be severed, and collecting all of the blood into 50 cc. centrifuge tubes by means of a funnel.

The serum may be allowed to separate from the clots or they may be broken up with a glass rod (my practice) and every drop of serum secured by centrifuging; even if some hemoglobin discolors the serum no harm has been done. To the unheated serum is now added an equal part of chemically pure glycerin and the mixture kept in a bottle or distributed in ampoules (2 cc. is equivalent to 1 cc. of serum). When kept in a refrigerator there is never any bacterial contamination since 50 per cent glycerin is bacteriostatic and gradually bactericidal. The titer drops a little within the first few days but thereafter remains remarkably constant over a period of years; Kilduffe[10] has reported one antisheep serum satisfactorily preserved by this method for seven years. The method is equally good for the preservation of anticattle and antihuman hemolysins although the latter are generally preserved dried on filter paper as described by Noguchi, by those who employ an antihuman hemolytic system.

For use in my test a 1 to 100 stock solution of the glycerolated antisheep serum is prepared as follows:

Serum-glycerin mixture	2 cc.
Saline solution	94 cc.
5 per cent phenol	4 cc.

This mixture can be kept in the refrigerator almost indefinitely since the 0.2 per cent concentration of phenol usually protects against contamination and the hemolysin keeps remarkably well; indeed Kilduffe[11] has found that when gross contamination is avoided the solution remains active, potent and practically unchanged in titer for a period of at least six months.

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CHAPTER XIII.

ADJUSTMENT OF THE HEMOLYTIC SYSTEM FOR THE COMPLEMENT-FIXATION TEST WITH SPECIAL REFERENCE TO THE KOLMER METHOD.

While the results of investigations have shown that guinea-pig serum is best adapted for complement in complement-fixation tests, *the amount to employ and the method of adjustment of the hemolytic system are factors of primary importance in relation to the specificity and sensitiveness of complement-fixation tests;* these subjects have required a large amount of attention devoted to comparative studies of different methods in order to arrive at conclusions concerning the best method from the standpoint of sensitiveness and accuracy.

PRINCIPLES INVOLVED.

The problem presents two main phases, namely, the "building up" of a hemolytic system and the method for routinely adjusting it for diagnostic tests. The investigator in this field cannot but feel great admiration for the work of Bordet, Wassermann, and other pioneers who must have conducted a great deal of research before announcing the relatively simple and highly efficient directions accompanying descriptions of their technique.

In *building up* a hemolytic system it is of prime importance to so adjust the quantities of corpuscles and hemolysin that the complement unit will never fall below a certain minimum because the amount or unit of complement required for complete lysis of a given amount of corpuscles may be reduced so greatly by an excess of powerful hemolysin as to render the unit entirely worthless in complement-fixation tests, owing to the anticomplementary effects of antigen and serum alone and the deterioration of complement during the period of primary incubation.

Under ideal conditions the *adjustment of the hemolytic system must be so conducted as to provide enough complement for these nonspecific agencies, a balance for specific fixation by antibodies and antigen, and a means for detecting and even measuring the latter for the purpose of specific diagnosis.*

Furthermore, the technique must adjust the variations of hemolytic activity and fixability of the complement, variation in the

sensitizing activity of the hemolysin and of resistance of the corpuscles to lysis.

Variation in hemolytic activity of complement, sensitizing activity of hemolysin and resistance of corpuscles are commonly adjusted by using a fixed and arbitrary amount of complement and corpuscles and titrating the hemolysin, as originally advocated by Wassermann and his associates; or, by using fixed and arbitrary amounts of hemolysin and corpuscles and titrating the complement. Many immunologists see no difference in the choice of either method and probably the majority follow the principles of Wassermann's technique, namely, daily titrations of the hemolysin; others claim that there is a difference in favor of the method of daily titrations of complement and an extensive series of comparative tests employing both methods has been required to settle the point in favor of the latter.

In order to allow for the anticomplementary effects of antigen and serum alone, Bordet, and later Wassermann, advocated the use of fixed amounts of complement which provided a considerable excess and probably resulted in reducing the sensitiveness of the reactions; subsequent investigators who titrated the complement, advised the use of but 1 or 2 units in order to avoid this excess and thereby increase the sensitiveness of the complement-fixation test. Recently others have sought to render the technique still more delicate by titrating the complement in the presence of antigen or of antigen and a normal serum, in order to make a closer adjustment for the anticomplementary activities of these substances.

Variation in fixability of guinea-pig serum complement is best overcome by using the mixed sera of several animals even though this involves the use of preserved complement over a period of about two or three weeks in order to avoid waste. Preliminary titration of complement for fixability by mixtures of extract and antibody serum is apparently practised by a few serologists and while this is advisable when the serum of a single animal is used, it is unnecessary with a mixture of sera and does not warrant the extra work and time involved, insofar at least as complement-fixation in syphilis is concerned.

In the titration of complement for hemolytic activity, the kind and duration of incubation exert an important influence upon the results and even the manner of mixing the three ingredients, namely, complement, hemolysin and cells. For example, water-bath incubation aids the hemolytic activity of complement more than thermostat (air) incubation and in setting up the titration, if the cells and hemolysin are mixed together for five minutes or more before the addition of complement, there is sufficient time for enough sensitization to occur to influence the results and reduce the unit of complement.

THE ABSOLUTE MINIMUM OF COMPLEMENT.

In every hemolytic system the amount of complement employed may be greatly reduced and complete hemolysis obtained by using increasing amounts of hemolysin as shown by Morgenroth and Sachs;[1] the converse is also true to even greater degree, that is, an excess of complement may produce complete lysis with less than the theoretical amount of hemolysin, which has an important bearing upon "building up" of a hemolytic system. The point of practical importance is that amounts of complement capable of producing complete hemolysis may be too large or too small for use in complement-fixation tests when used with 2 units of hemolysin, and particularly when the complement is used in a fixed amount and the hemolysin titrated. As shown in Table 8, varying dilutions of complement were used in constant amount of 1 cc. for titrating an antisheep hemolysin and employed in complement-fixation tests with 2 units of hemolysin; the unit of hemolysin became progressively higher as the amount of complement decreased, but the results of complement-fixation tests showed that the 1 to 10 dilution represented an excess of complement since the degree of fixation was masked by too much hemolysis; dilutions of complement higher than 1 to 40 represented too small an amount, the available complement being largely or entirely fixed in a non-specific manner by antigen and serum alone and resulting in inhibition of hemolysis in the serum controls and in tests conducted with normal serum. Similar experiments with an antihuman hemolytic system showed that while the unit of hemolysin may be readily obtained with progressive dilutions of complement used in constant dose of 0.1 cc., the higher dilutions may prove unsatisfactory. However, when the hemolysin is held as a constant and the complement titrated, the higher dilutions of complement may prove as satisfactory as the lower, the only difference being that larger amounts are used while the actual amount of complement in terms of undiluted serum remains about the same.

TABLE 8.—RESULTS OF COMPLEMENT-FIXATION TESTS CONDUCTED WITH AN ANTI-SHEEP HEMOLYTIC SYSTEM ADJUSTED BY USING VARYING DILUTIONS OF COMPLEMENT IN FIXED AMOUNTS WITH 2 UNITS OF TITRATED HEMOLYSIN.

Fixed amount complement.	Unit of hemolysin.	Syph. serum.		Syph. serum.		Normal serum.		Norman serum.	
		Antigen.	Control.	Antigen.	Control.	Antigen.	Control.	Antigen.	Control.
1 cc. 1:10	0.5 cc. 1:5000	+	−	−	−	−	−	−	−
1 cc. 1:20	0.5 cc. 1:3500	++++	−	+++	−	−	−	−	−
1 cc. 1:30	0.5 cc. 1:3000	++++	−	++++	−	−	−	−	−
1 cc. 1:40	0.5 cc. 1:2500	++++	−	++++	−	−	−	−	−
1 cc. 1:60	0.5 cc. 1:500	++++	+	++++	−	+	+	+	+
1 cc. 1:80	0.5 cc. 1:400	++++	++	++++	+	++	+	+	+

Without further discussion of this intricate and important subject, upon which a large amount of experimental data is to be found in literature, I wish simply to emphasize the practical difficulties encountered in building up an acceptable hemolytic system, the necessity for so adjusting the amounts of corpuscles and hemolysin that the average unit of complement will fall within a range neither too large nor too small, and that from this standpoint Matsunami, Rule and myself[2] have found that the hemolytic system is better adjusted by using the hemolysin as a constant and making daily titrations of complement than the converse.

COMPLEMENT VERSUS HEMOLYSIN TITRATIONS FOR THE DAILY ADJUSTMENT OF THE HEMOLYTIC SYSTEM.

As previously stated, Wassermann instituted the method of using complement in a fixed amount (0.1 cc.) and titrating the hemolysin for the purpose of adjusting an antisheep hemolytic system. This method is still used by numerous workers in Europe and America. An increasing number of others, however, have adopted a method of adjusting the hemolytic system by titrating the complement instead of hemolysin. Ottenberg and Frazier[3] have reported that this yields more delicate results and in their opinion the correct method for compensating for complements of greater than average hemolytic strength is by using a smaller dose of complement.

As a matter of fact a great deal of work has been required to settle the point for Matsunami, Rule and myself and especially so when comparative tests were made with single doses of patients' serum as 0.1 or 0.2 cc.; differences were, however, brought out by using each serum in graded amounts.

When tests were conducted with sera in amounts of 0.2 or 0.1 cc. the differences observed with the two methods were not nearly so striking; a résumé of such tests is given in Table 9 and shows a remarkable similarity of results observed with 236 sera insofar as positive or negative reactions were concerned, 179, or 76 per cent of sera, reacting positive in tests in which the hemolysin was titrated, and 183, or 78 per cent reacting positive in tests in which the complement was titrated. Of the positive reactions, the *latter method gave a higher percentage of strongly positive results, and the 4 sera reacting negative with titrated hemolysin and positive with titrated complement were from luetic individuals undergoing treatment.* Furthermore, in additional tests with syphilitic sera employed in amounts varying from 0.2 to 0.006 cc. more sensitive reactions were observed in those conducted with 2 units of titrated complement than in those employing a fixed amount of complement and 2 units of hemolysin. Therefore we reached the general conclusion that *superior results are observed when the complement is subjected to daily*

titration rather than the hemolysin, the differences in results being in the nature of more sensitive reactions and a slightly larger percentage of positive reactions with the sera of syphilitic individuals. As a *matter of fact the best results of all are obtained when both hemolysin and complement are subjected to daily titration.*

TABLE 9.—RESULTS OF WASSERMANN TESTS WITH 236 SERA CONDUCTED WITH TITRATED HEMOLYSIN AND CONSTANT AMOUNT OF COMPLEMENT AND TITRATED COMPLEMENT AND CONSTANT AMOUNT OF HEMOLYSIN.

Method.	Positive.	Negative.	Strongly positive.	Weakly positive.
Hemolysin titrated Complement constant	179	57	140	39
Complement titrated Hemolysin constant	183	53	172	11

THE TITRATION OF COMPLEMENT.

The method usually employed consists of titrating complement with 1 or 2 units of hemolysin and employing 2 units in the complement-fixation tests, in order to provide sufficient above and beyond that amount of complement neutralized by the anticomplementary properties of the antigen and serum alone.

Some investigators, believing that this amount of complement represents an excess, have modified the technique in order to elicit more delicate reactions. Thomsen[4] proposed to determine the free unit of complement by titrating in the presence of antigen and using in complement-fixation tests 1 unit of complement with 2 units of hemolysin. Boas[5] also titrates complement in the presence of the extract (antigen) after Thomsen's method, the unit being usually 0.08 cc. undiluted serum, and uses 1 unit in the main test with a fixed amount (0.05 cc.) for the serum control. Later McKenzie[6] Browning and McKenzie[7] proposed an ingenious method for measuring the anticomplementary activity of extract (antigen) and serum alone according to the amount of complement absorbed in units, and subtracting these from the amount absorbed by serum and antigen together. Still later Loyd Thompson[8] and Thomas and Ivy[9] proposed a method combining the principles of Thomsen's and McKenzie's methods consisting in titrating the complement in the presence of antigen and a normal serum to allow for the anticomplementary activities of these substances, and using 1 unit in the main tests with 1 unit of hemolysin.

There are, therefore, three methods for the titration of complement as follows:

(*a*) Titration of complement with hemolysin alone.

(*b*) Titration of complement in the presence of antigen.

(*c*) Titration of complement in the presence of antigen and normal serum.

After a thorough comparative study of these methods I arrived at the conclusion that *best results are secured by titrating the complement in the presence of the antigen and employing 2 full units in the conduct of the Wassermann and other complement-fixation tests when the primary incubation is eighteen hours at 3° to 6° C. as in my method.* This enables one to make an allowance for the anticomplementary action of the antigen and for a close adjustment of the hemolytic system without increasing sensitiveness to the point where non-specific reactions may occur.

The next best method was found to be the titration of complement in the presence of the antigen and a single or pooled negative serum. But this method was found to have certain objections, namely, (*a*) the necessity for using a serum absolutely free of antibody; (*b*) the necessity for using a serum free of anticomplementary activity and (*c*) the fact that the serum may contain enough natural anti-sheep or anticattle hemolysin to influence the titration unless it were removed by preliminary absorption with sheep or cattle cells. In practice it is easy to remove the natural hemolysins but it is risky to depend upon sera yielding negative Wassermann reactions in former tests and furthermore when one carries a normal serum or mixture over from week to week for the complement titrations it is almost sure to acquire some slight anticomplementary activity so that when the complement is titrated in its presence the unit is higher than it should be for testing fresh sera. Largely for this reason comparative tests have shown[10] that Wassermann, bacterial and other complement-fixation tests conducted with 2 full units of complement titrated in the presence of antigen alone, a primary incubation of eighteen hours at 3° to 6° C. followed by ten minutes in a water-bath and the addition of 2 units of hemolysin and corpuscles were more sensitive than tests conducted with 1 unit of complement titrated in the presence of antigen and normal serum, primary incubation of one hour in a water-bath and the addition of 1 unit of hemolysin and the corpuscles. The former therefore was selected as the method of choice in my method and the principles involved summarized as follows:

1. To so adjust the amounts of corpuscles and hemolysin that the unit of complement will fall within a range neither too large nor too small for the most sensitive and specific complement-fixation reactions. This range must be decided upon by actual experience with any given technique and may require the daily titration of hemolysin preliminary to the titration of complement in order to avoid using too much or too little hemolysin, as advised in my method.

2. Complement should be titrated in the presence of the test dose of antigen in order to allow for the non-specific fixation or absorption of complement by this reagent.

3. In titrating complement, the mixtures of complement and antigen should be placed in a water-bath at 38° C. for at least one hour before the addition of hemolysin and corpuscles.

4. After the addition of hemolysin and corpuscles a secondary incubation of one hour is advisable before reading the unit of complement, although experience may show that a safe reading may be made at the end of one-half hour.

5. For the syphilis complement-fixation test it is not necessary to make a preliminary titration of the fixability of complement *provided the mixed sera of at least 3 or more guinea-pigs are employed.* As stated on page 148 complement serum may be preserved for at least two weeks in order to avoid waste and this is to be preferred to the use of the serum of a single animal.

THE TITRATION OF HEMOLYSIN.

It is well known that exact quantitative relations between hemolysin and complement do not exist and are not mathematically progressive; curiously if the unit of hemolysin is determined with a fixed amount of complement, as, for example, 0.3 cc. of a 1 to 30 dilution, a back titration of the same complement with 1 unit of hemolysin will give a unit of complement smaller than the amount used for the titration of hemolysin, as, for example, 0.2 cc., and titration with 2 units of hemolysin reduces the unit of complement much more as, for example, to 0.15 cc. For the titration of hemolysin an arbitrary amount of complement must be used and the serum should be, of course, *a mixed serum from several guinea-pigs* in order to average the hemolytic activity of different complement sera and the content of natural hemolysin. Experience soon teaches what constitutes the average unit of complement best adapted for the complement-fixation technique being employed and this should be the fixed amount of complement employed for the titration of hemolysin in order to bring the units of complement sera on different days within the proper range. For example, if according to experience 0.1 to 0.4 cc. of a dilution of complement represents the proper range within which the unit of complement should fall in order to secure best results, the hemolysin should be titrated with 0.3 cc. of complement. *The point of practical importance is to use that amount of complement for the titration of hemolysin which will yield a unit of hemolysin which when employed for the routine and daily titration of complement will yield a unit of complement neither too large nor too small for the hemolytic system of the complement-fixation tests.*

Occasionally guinea-pig serum contains more than the average amount of natural antisheep hemolysin and when such serum is employed as complement the unit of hemolysin is reduced in amount

accordingly. For this reason I believe it is advisable to titrate the hemolysin with each complement serum rather than to use it in a fixed amount from week to week.

In the titration of hemolysin the mixtures of hemolysin, complement and corpuscles are incubated either in a water-bath at 38° C. or in a thermostat at the same temperature; the time allowed for hemolysis varies with different workers from fifteen minutes to two hours. Wassermann advised the use of the thermostat for one hour, but several investigators have stated that this period is unnecessarily long, and especially if a water-bath is employed. It is commonly believed that one-half hour in a water-bath is equal to one hour in a thermostat (air incubation) and the former has become widely adopted.

Miss Rule, Miss Trist and myself[11] found that the rate of hemolysis is greatly influenced by the amount of complement used, but in titrations which are strictly comparative, in order to elicit differences ascribable to the kind of incubation alone, the following have been observed: with water-bath incubation hemolysis is from 50 to 75 per cent completed at the end of one-half hour and generally completed or almost so at the end of one hour; with air incubation hemolysis is about 25 per cent complete at the end of one-half hour and about 90 per cent completed at the end of one hour.

From the standpoint of economy in time the water-bath is to be preferred; for the same reason and more especially since hemolysis is practically over in the majority of titrations at the expiration of one hour, *an incubation of one hour in a water-bath at 38° C. appears quite satisfactory for the titration of hemolysin* inasmuch as the absolute end-point in hemolysis is not necessary for a sufficiently close adjustment of the hemolytic system. The question of time is of more importance in relation to the titration of complement and should not be unduly shortened at the expense of accuracy and delicacy in results.

Very little or no difference in results of hemolysin titrations are found according to the manner of setting up hemolysin titrations provided two points are observed namely, (1) to add complement to the mixtures of corpuscles and hemolysin at once and (2) to shake the mixtures well and at once to prevent irregular sensitization of the corpuscles. If the titration is set up by mixing the corpuscles and hemolysin followed by an interval of five minutes or more before the addition of complement, there is sufficient time for some sensitization of the cells which results in a smaller unit of hemolysin for complete hemolysis than observed when complement is added at once and before this preliminary sensitization occurs. If the corpuscles are added very slowly or if the mixture of hemolysin, corpuscles and complement are not shaken, a portion of the corpuscles may absorb more than their share of hemolysin and yield

an irregular and confusing result. Too great dilution with saline solution and the use of too narrow tubes giving a high column of fluid are to be avoided because they are likely to produce irregular results.

THE AMOUNTS OF COMPLEMENT AND HEMOLYSIN TO EMPLOY IN COMPLEMENT-FIXATION TESTS.

The proper amount of complement to employ in the Wassermann and other complement-fixations tests is the smallest amount *yielding the most sensitive specific reactions.* It varies greatly with the technique employed. The dose should provide enough for allowing for the anticomplementary action of antigen alone, serum alone and for deterioration during the primary incubation with enough left over for specific fixation by antigen and antibody if the latter is present in the serum or spinal fluid being tested.

In the method, which employs a primary incubation of eighteen hours at 3° to 6° C. followed by ten minutes in a water-bath at 38° C., *experience* has shown that the best results are secured by using 2 full units along with 2 units of hemolysin, the complement being titrated in the presence of the antigen. More sensitive reactions can be obtained by using less complement or less hemolysin but in the long run the tests are unsatisfactory because of anticomplementary reactions and the danger of non-specific results. However, tests conducted with 2 *exact* units of complement and 2 units of hemolysin are generally satisfactory with fresh sera and spinal fluids and the reactions are slightly more sensitive than in those employing 2 *full* units (see Chapter XXXI for difference between the exact and full units); tests conducted with 2 full units of complement and 1 unit of hemolysin are also satisfactory with the majority of sera and the reactions more sensitive but when 2 exacts units of complement are used with 1 unit of hemolysin the hemolytic system is too close and the incidence of incomplete hemolysis of the controls too high. It has been my aim to evolve a test specific for syphilis in those countries where yaws and trypanosomiasis do not occur and I think this ideal has been attained by refusing to make the test too sensitive by using too small an amount of complement; therefore I have chosen to use 2 full units along with 2 units of hemolysin and have sought to increase sensitiveness by using a very sensitive antigen and the eighteen-hour refrigerator incubation.

The amount of hemolysin employed by different serologists in the conduct of complement-fixation tests for syphilis varies to a considerable extent and especially among those using antisheep and antiox hemolytic systems. A few employ a single unit but the

majority use 2 or more units; apparently all workers employing the antihuman hemolytic system use 2 units.

As is now well known an excess of hemolysin under certain conditions may produce complete hemolysis even though a portion of the complement has been fixed by syphilis antibody and antigen, thereby yielding falsely negative reactions with weakly positive sera (but not with strongly positive sera); this is especially apt to occur when the tubes are placed in a refrigerator over night for the settling of corpuscles before the reactions are read. While it is customary to hold this fact as a weighty argument against the antisheep hemolytic system owing to the influence of natural antisheep hemolysins, recent studies by Williams[12] have shown that natural antihuman hemolysins may have a similar influence upon an antihuman hemolytic system unless special precautions are taken in the choice of corpuscles.

Allowance for non-specific fixation of complement by antigen and serum alone is made by using an excess of complement or hemolysin or both; as stated above I arrived at the conclusion that best results are secured by titrating the complement in the presence of antigen and using 2 units. Under these conditions the proper amount of hemolysin to employ is that which will result in complete hemolysis of all controls and tests with non-syphilitic sera within the hour of secondary incubation, but fail to produce hemolysis or give but partial hemolysis, when the complement has been fixed by syphilis antibody and antigen. In other words the problem is to avoid an excess of immune hemolysin and neutralize the influence of natural hemolysins for the purpose of detecting the slightest degree of specific fixation of complement.

An excessive dose of hemolysin may be harmful both during and after the secondary incubation. It may greatly hasten hemolysis and produce falsely negative reactions with weakly positive sera or at least mask the actual degree of specific fixation of complement when a fixed period of secondary incubation is being used, as one hour in a water-bath. By continuing hemolysis after the secondary incubation the same effects may be produced although these may be prevented by reading the reactions within two or three hours or by the very simple procedure of placing the tubes in a water-bath at 55° C. for ten minutes (not longer) which inactivates all traces of complement and thereby breaks up the hemolytic system.[13]

Theoretically at least the best work, that is, the most sensitive specific reactions, require that there should be no natural hemolysins in the patients' sera or complement to interfere with a close adjustment of the hemolytic system.

Routine removal of natural hemolysins from sera would appear to be the only method for obtaining these conditions with an antisheep hemolytic system and I have adopted this practice.

Another method employed by some serologists is to carefully watch each serum control during the secondary incubation and remove the tubes for reading as soon as the controls show complete hemolysis.

The influence of natural hemolysin in the complement serum may be adjusted by the simple procedure of titrating the immune hemolysin each day with an average amount of complement, as in my method. If the complement happens to be rich in natural hemolysin a correspondingly smaller amount of immune hemolysin will be found to constitute a unit; if the complement is free of natural hemolysin the unit of immune hemolysin will be correspondingly greater.

In methods employing a fixed and arbitrary dose of complement as in the original Wassermann test, the unit of hemolysin is easily determined each day by titration with this fixed dose of complement. In methods employing a titration of complement, the hemolysin should be titrated with the average unit of complement as determined by experience. Some serologists have apparently overlooked this important detail and determine the "unit" of hemolysin with an amount of complement out of proportion to that used in their complement-fixation tests. Under these conditions what is designated as a "single unit" may actually be more or less than a unit in the main tests.

This is especially true if less than the average unit of complement is employed in the titration. Under these conditions a larger amount of hemolysin must be used for a unit and may represent an excess when employed in complement-fixation tests with larger amounts of complement.

The use of an excessive amount of hemolysin in the complement titration may reduce the unit of complement to such a small amount of serum as to be insufficient for complement-fixation tests even when used in 2 units, that is, the unit of complement is below the absolute minimum. In the experience of Yagle, Rule and myself[14] *best results have been secured by titrating the hemolysin with an average unit of each complement serum; this is followed by a titration of the complement in the presence of antigen.*

We believe that it should be a fixed principle that *the hemolysin be used in the same amount for both the complement titration and the main complement-fixation tests.* Some serologists titrate the complement with a fixed amount of hemolysin (1 or 2 units) but use a larger amount of hemolysin with 2 units of complement in the complement-fixation tests; this practice results in the use of an excess of hemolysin and reduces the sensitiveness of the tests.

In my method the corpuscles are used in 0.5 cc. of a 2 per cent suspension and the average amount of complement used in the hemolysin titration is 0.3 cc. of a 1 to 30 dilution. The hemolysin is

titrated with the complement serum of each day's work to adjust for any possible influence of natural hemolysin in the complement serum. The extra work and time involved are almost negligible and more than compensated for by the improvement in results.

The complement is then titrated in the presence of antigen; the question arises how many units of hemolysin should be employed?

If 1 unit of hemolysin is used the unit of complement represents more serum than if 2 or more units are employed. If 4 to 6 or more units of hemolysin are used the unit of complement is correspondingly smaller and may reach an amount so small that even 2 units are less than the absolute minimum and entirely unsatisfactory for complement-fixation tests.

As shown in Table 10 the average unit of 14 different complement sera was 0.26 cc. of 1 to 30 dilution when titrated with 1 unit of hemolysin but 0.2 to 0.19 cc. when titrated with 4 or more units of hemolysin. Experience has shown that under these conditions a complement unit of 0.15 cc. or less serum is unsatisfactory even when used in a dose of 2 units; as shown in the table the use of 4 to 6 units of hemolysin is likely to reduce the unit of complement to this unsatisfactory absolute minimum with a large percentage of complement sera.

Experience has also shown that complement-fixation tests conducted with 2 units of complement based upon titrations with 1 and 5 units of hemolysin yield the most delicate reactions with antisheep and antiox hemolytic systems; both methods, however, are unsatisfactory because of too close adjustment of the hemolytic system and without sufficient allowance for non-specific fixation of complement by serum alone and antigen alone in the ice-box method of primary incubation.

TABLE 10.

Complement sera 1 to 30	Units of complement* with			
	1 unit hemolysin.	2 units hemolysin.	4 units hemolysin.	6 units hemolysin.
1	0.30	0.30	0.25	—
2	0.20	0.15	0.15	—
3	0.20	0.15	0.15	0.10
4	0.35	0.35	0.30	0.30
5	0.25	0.25	0.25	0.25
6	0.30	0.30	0.25	0.25
7	0.25	0.20	0.20	0.20
8	0.20	0.15	0.15	0.10
9	0.20	0.20	0.15	0.15
10	0.20	0.20	0.15	0.15
11	0.20	0.20	..	0.15
12	0.35	..	..	0.25
13	0.25	0.20	..	0.15
14	0.35	0.30	..	0.25
Average	0.26	0.23	0.2	0.19

* Each complement serum was a mixture of the sera of three or more guinea-pigs.

With the antihuman and antichicken hemolytic systems 1 unit of hemolysin is sufficient because the unit of complement represents too much guinea-pig serum and greatly reduces the sensitiveness of complement-fixation tests.

The sum total of a large number of experiments has proved for us that *best results are observed by titrating the complement with 2 units of hemolysin and conducting the complement-fixation tests with 2 units of complement and 2 units of hemolysin.*

For example, in tests conducted with 1 unit of hemolysin for the complement titrations and main complement-fixation tests, the percentage of reactions showing incomplete hemolysis of the serum and antigen controls was highest, and least in reactions employing 2 units of hemolysin for the complement titrations and main tests. Five units of hemolysin gave fewer of these anticomplementary reactions than observed with 1 unit of hemolysin but more than observed with 2 units.

If the serologist could always use perfectly fresh human sera, 1 unit of hemolysin for the complement titration and main tests would be satisfactory, but if older sera must be used and if he insists upon *perfectly hemolyzed controls,* best results eventually follow the use of 2 units of hemolysin. It is emphasized that these observations are based upon a primary incubation of eighteen hours at 8° C. which increases the degree of non-specific fixation of complement by sera and antigens alone as well as by mixtures of these.

With 1 and 5 units of hemolysin for the complement titrations and main tests the controls of some sera require more than one hour for complete hemolysis; hemolysis with 1 unit is usually slower than with 5 units and quickest with 2 units, Very rapid or "explosive" hemolysis, which some serologists desire, is apt to reduce the sensitiveness of reactions with weakly positive sera, but fairly rapid hemolysis which is complete in one-half to one hour appears to be desirable and yields very delicate reactions if read within two or three hours after removal from the water-bath.

With antisheep and antiox hemolytic systems the most sensitive reactions were usually observed when the complement was titrated and the complement-fixation tests conducted with 1 unit of hemolysin and if it were not for the fact previously discussed that the serum controls not infrequently show incomplete hemolysis with 1 unit of hemolysin, the use of a single unit would be the method of choice with these hemolytic systems but in order to avoid non-specific reactions and unsatisfactory controls we have concluded that *best results are secured when 2 units of hemolysin are used for the complement titrations and main tests. The differences in sensitiveness of reactions are very slight and the results are uniformly more satisfactory.*

SENSITIZED VERSUS PLAIN RED BLOOD CORPUSCLES IN COMPLEMENT-FIXATION TESTS.

In the technique of complement-fixation tests it is the usual custom to add hemolysin and cells separately after the primary incubation; in some laboratories, however, the hemolysin and cells are mixed and allowed to stand at varying temperatures for varying periods of time and the "sensitized" corpuscles added. This practice is particularly in vogue with the antisheep and antiox hemolytic systems, but not with the antihuman, because the presence of hemagglutinins in rabbit antihuman serum generally interferes with sensitization and the use of sensitized corpuscles.

The use of sensitized corpuscles decreases somewhat the amount of work connected with the conduct of complement-fixation tests and generally yields more rapid and sharper hemolysis, as compared with the usual method of adding corpuscles and hemolysin separately; *but of greater importance is the effect produced upon the sensitiveness and accuracy of the tests as compared with the method of adding corpuscles and hemolysin separately.*

Various methods have been proposed for the sensitization of corpuscles involving the amount of hemolysin used, the duration of sensitization and the temperature of exposure, all of which have some bearing upon the results of complement-fixation tests conducted with sensitized corpuscles.

Sensitization is a very rapid process and particularly at ordinary room temperature (about 20° C.), being generally more rapid at this temperature than in a water-bath at 38° C. or in a refrigerator at 6° to 8° C. For the absorption of 1 unit of hemolysin, one-half hour at room temperature is generally sufficient; for the absorption of 2 to 10 units at least forty-five minutes to one hour should be allowed.

Insofar as the results of complement titrations are concerned the maximum of sensitization is reached when sheep corpuscles are sensitized with 4 to 6 units of hemolysin, that is, corpuscles exposed to larger amounts of hemolysin do not become more sensitive to the hemolytic activity of complement. Morgenroth and Sachs[15] have also found that sheep cells sensitized with at least 5 units of hemolysin represented the maximum degree of sensitization for guinea-pig complement.

That sheep corpuscles, however, may absorb larger amounts of hemolysin and as much as 12 to 20 units at least, is indicated by the results of tests for unabsorbed hemolysin after the corpuscles have been removed by centrifuging; *the excess of hemolysin above 4 to 6 units, however, does not increase the susceptibility of sheep corpuscles to the hemolytic activity of guinea-pig complement.*

It is well known that sensitized corpuscles are decidedly more sus-

ceptible to the hemolytic activity of complement than plain corpuscles exposed to the same amounts of hemolysin and complement; therefore the amount of complement required for the complete lysis of sensitized corpuscles is always less than that required for an equal amount of plain corpuscles in the presence of the same amount of hemolysin. This fact is of fundamental importance in relation to the use of sensitized corpuscles for the conduct of complement-fixation tests when the complement is titrated with plain corpuscles and the tests conducted with sensitized corpuscles; under these conditions the hemolytic system may not be closely enough adjusted and tests conducted with corpuscles sensitized with 1 to 6 units of hemolysin are apt to be correspondingly less sensitive, because the amount of complement is excessive. For example, 1 unit of complement titrated with plain corpuscles and 1 unit of hemolysin may be 0.25 cc., but only 0.2 cc. when titrated with corpuscles previously sensitized with the same amount of hemolysin, 0.15 cc. when the corpuscles are sensitized with 2 units of hemolysin and 0.09 cc. with corpuscles sensitized with 5 units of hemolysin.

In tests conducted according to the principles of Wassermann's technique, namely, with a constant amount of complement and 2 units of titrated hemolysin, the use of corpuscles previously sensitized with the hemolysin yields less sensitive reactions than tests in which the corpuscles and hemolysin are added separately. The reason is given above, namely, that sensitized corpuscles require less complement for complete lysis than the same amount of plain corpuscles in the presence of the same amount of hemolysin added separately.

These differences are most apparent in comparative tests employing graded amounts of each serum; when large single doses of serum are employed the differences in degree of sensitiveness may not be apparent with strongly positive sera, but are strikingly so with weakly positive sera. Furthermore, the differences are more apparent with plain than with cholesterolized antigens, because the latter absorb more complement and yield stronger complement-fixation reactions.

But when the complement is titrated with the same sensitized corpuscles as used in the complement-fixation tests the reactions are more nearly equal in sensitiveness to those conducted with plain corpuscles. The reason is apparent because the unit of complement adjusted to sensitized corpuscles is smaller than the unit obtained with plain corpuscles and an excess of complement is thereby avoided in conducting the complement-fixation tests. While tests conducted with sensitized corpuscles may be of equal or even greater delicacy than duplicate tests conducted with plain corpuscles, *generally the tests conducted with sensitized corpuscles are somewhat less sensitive.* A great deal depends upon the size of the unit of comple-

ment, but when 2 units are employed in complement-fixation tests sensitized corpuscles are more susceptible to the hemolytic activity of any free or unfixed complement than plain corpuscles, and consequently show a somewhat greater degree of hemolysis.

These differences in sensitiveness of reactions are best demonstrated in tests employing graded amounts of serum; when but one dose of serum is employed the results may be strikingly similar. But if complement-fixation tests are conducted with sensitized corpuscles for the sake of convenience, it is imperative to titrate the complement rather than to use a constant or fixed dose, and furthermore, *the complement should be titrated with the sensitized rather than with plain corpuscles.*

Of additional importance in relation to a decision upon the use of plain or sensitized corpuscles for complement-fixation tests is the probable influence of natural hemolysins in the sera being tested upon the occurrence and degree of hemolysis. In other words, are tests conducted with sensitized sheep corpuscles subject to the influence of natural antisheep hemolysin to the same degree as tests conducted with plain corpuscles?

As stated above, when sheep corpuscles are sensitized with 4 to 6 units of hemolysin, the maximum degree of susceptibility to the hemolytic activity of complement is reached; accordingly it would appear that in tests conducted with corpuscles sensitized with 4 to 6 units of hemolysin, an excess of free hemolysin in the patient's serum would not enhance the degree of hemolysis. Actual experiments by Rule, Trist and myself[16] have shown that this may be true. In our experiments the degree of hemolysis with corpuscles sensitized with 1, 2 and 3 units of hemolysin was sometimes increased in the presence of free or additional hemolysin, whereas with corpuscles sensitized with more than 4 units of hemolysin the degree of hemolysis was not usually influenced by the presence of as much additional hemolysin as 6 units, which is much greater than the amount encountered in the form of natural antisheep hemolysin in an individual's serum. The results showed therefore *the advisability of sensitizing corpuscles with 4 to 6 units of hemolysin instead of 1 or 2 if sensitized corpuscles are employed in complement-fixation tests, in order to reduce the influence upon hemolysis of free or natural hemolysin in human sera.*

This naturally brings up the question whether or not it is possible to avoid the influence of natural antisheep hemolysin in human sera by using corpuscles sensitized with 4 to 6 units of hemolysin. Off hand this would appear to be true but as a matter of fact a large number of comparative tests have proven to us that contrary to expectations and hopes, *tests conducted by adding corpuscles and hemolysin separately have yielded more sensitive reactions insofar as my method is concerned.*

For example Miss Yagle and I found that if the complement is titrated with plain sheep corpuscles as usual and the corpuscles and hemolysin (2 units) mixed for an hour and added together after the primary incubation in order to save the little extra time of adding them separately, the reactions were distinctly less sensitive than when hemolysin and corpuscles were added separately. Even when the corpuscles were sensitized with 2 units of hemolysin for the complement titration and main tests the reactions were much less sensitive than duplicate tests conducted in the usual manner by adding hemolysin and corpuscles separately. In both methods from 70 to 80 per cent of reactions with syphilitic sera were weaker in quantitative tests employing sensitized cells although the percentage of positive reactions were practically the same as observed when hemolysin and corpuscles were added separately. The percentage of anticomplementary reactions with sensitized cells was slightly less.

Practically the same results have been observed with sheep corpuscles sensitized with 5 units of hemolysin. Of particular interest were the results of comparative tests with sera before and after removal of natural antisheep hemolysin because the main purpose of using sensitized corpuscles would be to save the labor and time involved in the absorbing sera routinely before testing, providing nothing was lost in sensitiveness even if nothing more were to be gained. The results of hundreds of comparative tests have forced Miss Yagle and myself to the conclusion, however, *that the best and most sensitive reactions in syphilis are observed when the natural antisheep hemolysin is removed and the tests conducted by the usual method of adding corpuscles and hemolysin separately.* Taking the sera as tested routinely at the Polyclinic, Philadelphia, and therefore including those *from syphilitic and non-syphilitic individuals* the results have been approximately as follows:

1. Forty-two per cent positive when natural antisheep hemolysin was not removed and the sera tested with plain corpuscles.

2. Thirty-three per cent positive when natural antisheep hemolysin was not removed and the sera tested with sensitized corpuscles.

3. Seventy-five per cent positive when natural antisheep hemolysin was removed and the sera tested with plain corpuscles (my routine method).

4. Forty-two per cent positive when natural antisheep hemolysin was removed and the sera tested with sensitized corpuscles.

In a second series of comparative tests with a series of 35 sera from syphilitic and non-syphilitic individuals conducted at the same time with the same reagents, essentially similar results were observed as follows:

1. Twenty-three per cent positive reactions with plain sera and sensitized corpuscles.

2. Thirty-two per cent positive reactions with hemolysin-free sera and plain corpuscles (my routine method).

3. Thirty per cent positive reactions with plain sera and plain corpuscles.

4. Tests conducted with hemolysin-free sera and sensitized corpuscles showed so many instances of incomplete hemolysis with normal sera that the percentage of positive reactions with syphilitic sera could not be accurately stated.

All of the postive reactions in both series occurred with syphilitic sera; none were non-specific. The corpuscles were sensitized with 5 units of hemolysin and employed in the complement titrations as well as in the main tests. But the reactions were unquestionably most sensitive in tests employing plain corpuscles and the best results were observed in our routine method which employs hemolysin-free sera tested with plain corpuscles. This was found true not only in relation to the absolute percentage of positive reactions but also in the degree of positiveness from the quantitative standpoint. Lyne[17] has recently reported that 1000 comparative tests by Ecker's[18] method employing sensitized corpuscles (5 units) and my method which employed plain corpuscles and sera from which the natural antisheep hemolysin had not been removed, showed both of equal sensitivity and specificity and that the former modification is uninfluenced by natural antisheep hemolysin. But in a recent comparative study of different methods and antigens by Dr. Ruth Gilbert and Miss Virginia Langworthy for the American Public Health Association, consisting of sending portions of each specimen of blood to seven different laboratories including the Western Reserve University School of Medicine and my own, the reactions reported by my laboratory on the basis of tests with hemolysin-free sera and plain corpuscles were found to be considerably more sensitive than those reported from Cleveland employing Ecker's test.

Therefore I am forced to the conclusion that *insofar as my test is concerned the best and most sensitive reactions are to be observed when the natural antisheep hemolysin is removed from all sera and the tests conducted with plain corpuscles and 2 units of hemolysin added separately* as I described in 1923. I wish it were otherwise as I would prefer any modification tending to shorten and simplify the method but I am unwilling to accept any change that involves or even threatens the principle of the maximum of sensitiveness consistent with specificity.

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CHAPTER XIV.

ANTIGEN FOR THE SYPHILIS COMPLEMENT-FIXATION (WASSERMANN) TEST WITH SPECIAL REFERENCE TO THE KOLMER ANTIGEN.

Probably the most important single factor influencing the results and practical value of the complement-fixation test in syphilis concerns the kind of organ extract employed as "antigen," and the divergent Wassermann reactions observed in different laboratories with portions of the blood of the same person have been due in large part to the difference in antigens employed, referable not only to a difference in kind but to dosage as well.

Historical Review.—As is now well known the first "antigens" employed by Wassermann, Neisser and Bruck[1] and Detre,[2] were salt solution extracts of syphilitic tissues, pure cultures of *Spirochæta pallida* not being available for their preparation. With these extracts the reactions were considered biologically specific, that is, a fixation of complement by antibody and specific pallida antigen.

No one supposed that the mechanism was entirely different until Marie and Levaditi[3] and Landsteiner, Müller and Pötzl[4] showed that similar extracts of non-syphilitic tissues and, indeed, alcoholic extracts of guinea-pig heart, served equally well as "antigen" in the Wassermann test. This ushered in the second period of development of the Wassermann reaction, stimulating research activity and the publication of numerous reports confirming the observation that extracts of normal organs may be used as "antigen" and that the reaction was not biologically specific in the sense of Bordet-Gengou's phenomenon.

One result of these researches was to show that the real antigenic substances in both salt solution and alcoholic extracts of either syphilitic or normal tissues were lipoidal substances and much work was done on the identification of these by Levaditi and Yamanouchi,[5] Porges and Meier,[6] Sachs and Altman,[7] Fleishmann,[8] Noguchi,[9] Hessberg[10] and others. Without detailing the large amount of investigation on this subject, the net results showed that many lipoidal substances were concerned, that no one was as good as a mixture and that synthetic "antigens" were generally unsuccessful. Noguchi's work was continued for several years with the aid of Bronfenbrenner[11] and proved particularly fruitful, having resulted in the discovery that the phosphatids, and particularly lecithins, were highly antigenic, a method being worked out for preparing these from tissues and now well known as the extracts of acetone insoluble lipoids.

While numerous researches had shown that alcoholic solutions of cholesterol were but feebly antigenic, Browning, Cruickshank and McKenzie[12] found that the addition of cholesterol to solutions of lecithins greatly improved their antigenic value; Sachs[13] found that the addition of cholesterol to alcoholic extracts of heart muscle likewise improved antigenic values and these discoveries may be said to have ushered in a third epoch in the development of "antigens" for the Wassermann test. Sachs' work was quickly confirmed in England by McIntosh and Fieldes[14] and in this country by

Walker and Swift[15] and by Laubaugh, Williams and myself.[16] These cholesterolized extracts have come into general use and are regarded by Field,[17] Kilduffe[18] and other serologists as approaching closest to the ideals of a standard antigen.

Certainly the phrase "specific antigen" cannot be applied to the extracts in common use; very probably salt solution extracts of syphilitic tissues (Wassermann's and Detre's original antigens) may contain specific products of pallida, but this is doubtful of alcoholic extracts. Following the successful cultivation of *Spirochæta pallida* Noguchi[19] prepared saline extracts and secured positive complement-fixation reactions with the sera of some syphilitic individuals, but these antigens have not proved of practical value. Craig and Nichols[20] found that alcoholic extracts of *Spirochæta pallida* gave a large percentage of negative reactions with syphilitic sera; Williams, Laubaugh and myself[21] found that both saline and alcoholic extracts were but feebly antigenic and particularly the latter, and that the reactions observed may be due to the extraction of sufficient lipoidal substances of non-specific character from the microparasites. Hinkleman[22] has more recently revived interest in the subject and believes that saline suspensions of cultures of *Spirochæta pallida* may be employed in complement-fixation tests for syphilis but in my experience the reactions are too irregular and weak with syphilitic sera and very much inferior to those obtained with tissue extracts, both in the diagnosis and as a serological guide in the treatment of syphilis.

PROPERTIES OF TISSUE EXTRACT ANTIGENS.

Salt solution and alcoholic extracts of tissues possess the following three well-known properties in relation to complement-fixation tests: hemolytic, anticomplementary and antigenic activities. *Hemolytic activity* refers to the property of the extracts in producing hemolysis of corpuscles either alone or in the presence of serum; *anticomplementary activity* refers to the property of the extracts in producing the destruction or inactivation of complement either alone or in the presence of normal serum; *antigenic activity* refers to the property of the extracts in "fixing" or inactivating complement in the presence of antibody in syphilitic serum. It may be stated that extracts vary greatly in these properties and the most desirable are those possessing high antigenic and low anticomplementary and low hemolytic activity.

The investigations of Noguchi, Erlandsen, Browning and McKenzie, Neyman and Gager and others, have indicated that the properties of various substances commonly found in alcoholic and ethereal extracts of tissues may be summarized as follows:

Proteins and cleavage products of proteins	Slightly hemolytic
Cholesterol and unidentified lipoidal bodies	Highly anticomplementary
Various salts	Slightly antigenic
Soaps and neutral fats	Highly hemolytic
Saturated and unsaturated fatty acids	Slightly anticomplementary
Bile salts (in extracts of liver)	Slightly antigenic
Phosphatids (especially lecithin); diaminomonophosphatids	Slightly hemolytic
	Slightly anticomplementary
	Highly antigenic

The ether and alcohol soluble but acetone insoluble constituents of tissues (lecithins) therefore are most antigenic and most desirable as antigens; on the other hand, brain and egg-yolk lecithins are comparatively inactive. Cholesterol itself in colloidal solution is highly anticomplementary and but feebly antigenic although when added to alcoholic extracts in small amounts (0.1 to 0.2 per cent) it greatly increases their antigenic sensitiveness.

THE IDEAL EXTRACT ANTIGEN.

1. It is apparent that extracts employed as antigens in the syphilis complement-fixation test should be as *highly antigenic and as little hemolytic and anticomplementary as possible;* the dose corresponding to 5 to 10 antigenic units should be at least ten times less than the anticomplementary and hemolytic units and especially with the refrigerator method of primary incubation, in order to conduct a sensitive test and yet remain free of the suspicion of yielding nonspecific reactions. In other words, the antigenic *unit* should be at least *twenty to forty times* less than the anticomplementary and hemolytic units and especially if cholesterol has been added.

2. The extract should be *highly polytropic;* this means that it should possess affinity for the lipodophilic antibodies in the sera of all syphilitics. As a result of routinely conducting the Wassermann test with three or more extracts during a period of eight years, I have frequently encountered sera containing an antibody with greater affinity for one extract than for others, all extracts being employed in a dose of 2 antigenic units. These results have been ascribed to the presence in a particular extract of a lipoidal substance having a peculiar or marked affinity for the lipodotropic syphilis antibody of a particular serum and the subject has not received the attention of other serologists warranted by its practical importance. *The addition of cholesterol to extracts renders them highly polytropic and constitutes one of the real values of cholesterolized extracts.*

3. The extract should *keep well* for several months at least; it is not uncommon for an extract to keep practically unchanged for a year or more while others may become highly anticomplementary in a week or month.

4. The process of making the extract should be so standardized that different extracts may possess approximately the same properties; any method yielding only occasionally an acceptable extract is not satisfactory.

Of course cheapness and simplicity of manufacture are likewise desirable features of any method, but these are certainly of much less importance; *quality must be recognized as of prime importance, as so*

much of the real value of the Wassermann test, namely, for the diagnosis of the obscure rather than the clinically manifest case of syphilis, depends upon the goodness of the extract employed as antigen.

KIND OF TISSUE EXTRACTED IN RELATION TO ANTIGEN FOR THE WASSERMANN TEST.

Naturally a large number of different tissues have been extracted by various workers for the preparation of antigens; probably the most widely used have been normal and syphilitic human liver, human, beef and guinea-pig heart, beef liver and guinea-pig liver and spleen. Simonelli has used syphilitic cornea of rabbit; Desmonliere beef tonsils and Baeslack syphilitic testes of rabbits, but none of these have been generally adopted.

It would appear that the majority of serologists have found extracts of non-syphilitic tissues equal or superior to extracts of syphilitic liver as antigens for the Wassermann test; the only extracts which may merit the designation of "specific" in the biological sense, are saline rather than alcoholic extracts of pure cultures of *Spirochæta pallida* or of tissues containing large numbers of these, and both, particularly the former, are not as satisfactory for the conduct of the Wassermann test as alcoholic extracts of tissues.

Trist and myself[23] have prepared and tested alcoholic extracts of the brain, heart, lung, liver, kidney and thigh muscles of human beings, rabbits and guinea-pigs; all tissues were used fresh, minced to the same degree, used in the same proportions, extracted at the same time and in the same incubator and titrated at the same time for hemolytic, anticomplementary and antigenic activities at varying intervals.

The results of these experiments have shown the following:

1. Rabbit tissues are decidedly inferior to those of human beings and guinea-pigs for the preparation of extracts.

2. Heart, liver and kidney tissue proved best for the preparation of extracts, named in the order of preference. Extracts of the brain, lung and muscle proved very poor from the standpoint of antigenic activity.

3. Extracts of fresh beef heart were generally more satisfactory than corresponding extracts of human heart; extracts of human muscle which had undergone postmortem changes were unsatisfactory owing to high degree of hemolytic activity. Extracts of human heart muscle generally acquired more anticomplementary substances and more quickly than corresponding extracts of beef hearts. These differences may have been due to the fact that the beef hearts were perfectly fresh and used within a few hours after removal. Extracts of different beef and human hearts prepared at the same time and in

exactly the same manner, varied to a slight degree in antigenic activity. Weiss[24] has prepared antigens by extracting vegetable materials; I have had no experience with them. Miss Yagle and I have observed that antigens prepared by hog heart muscle were generally less antigenic than antigens of beef heart.

4. *Extracts of guinea-pig heart were usually highly anticomplementary* and inferior to extracts of beef and human heart. Furthermore as stated on page 70 alcoholic extracts of guinea-pig heart, liver and spleen are objectionable from the standpoint of the possibility of giving positive complement-fixation reactions with the heterogenetic antisheep antibody (hemolysin) present in such a large number of human sera. Georgi, Taniguchi, Hooker and others have drawn particular attention to this point so that alcoholic extracts of guinea-pig heart are not only inferior antigens from the standpoint of relatively high anticomplementary activity but likewise and more importantly from the standpoint of possibly yielding positive nonspecific Wassermann reactions with this natural heterogenetic antibody in human sera. The same objection pertains to extracts of tissues from the horse and cat while those of human beings, rabbit, cattle and sheep are devoid of the property. Insofar as horse extracts are concerned I may also mention that in a study of them in my laboratory by Strumia it was observed that plain and cholesterolized extracts as well as the acetone insoluble lipoids were slightly inferior to corresponding extracts of beef heart prepared at the same time and in the same manner.

Of the tissues included in this study *extracts of beef and human heart have proved most satisfactory;* Noguchi, Browning and McKenzie and others have found beef liver satisfactory for the extraction of lecithins, but for plain alcoholic extracts heart muscle is to be preferred. Ruediger[25] found extracts of dog and guinea-pig heart highly anticomplementary or, at least, especially likely to yield nonspecific reactions. In our experience *fresh beef heart muscle (fat free) has been found generally most satisfactory for the preparation of plain alcoholic extracts and especially mixtures of the muscle of several hearts, which is readily obtainable if dried muscle powder is employed.*

A COMPARISON OF METHODS FOR THE EXTRACTION OF TISSUES IN THE PREPARATION OF ANTIGEN.

Alcohol versus Salt Solution Extracts of Tissues.—It has been stated but not proved, that salt solution extracts of syphilitic and nonsyphilitic tissues contain the same lipoidal substances as alcoholic extracts, although the quantities may be different. Saline extracts are apparently still used and especially in some European laboratories.

Comparative tests with saline and alcoholic extracts of the same tissues prepared at the same time and under identical conditions have shown in the experience of Trist and myself that *alcohol serves for the extraction of more antigenic substances than salt solution* regardless of the tissue employed and the duration and temperature of extraction. Those laboratories employing saline extracts usually follow Wassermann's custom of determining the anticomplementary unit and using one-half this amount for the main tests; but this principle and practice are wrong, inasmuch as there is no constant relation between anticomplementary and antigenic activities and an extract may be anticomplementary and yet almost devoid of antigenic sensitiveness.

Alcohol versus Ether Extraction.—As previously stated, the investigations of Neymann and Gager and others have shown that ether extracts from wet and dried tissues lipoidal substances which are not only anticomplementary and feebly antigenic but likewise highly hemolytic; Noguchi purified these ether extracts, that is, largely removed the anticomplementary and hemolytic substances, by precipitating with acetone and recovering the highly antigenic lecithins in the residue.

Our experiments, consisting of the extraction of similar amounts of wet and dried tissues and especially human and beef heart with ether and absolute ethyl alcohol under identical conditions, have usually shown that the *ether extracts were highly hemolytic* although the anticomplementary and antigenic values were usually similar to those of the alcoholic extracts; furthermore, alcohol usually extracted more lipoidal and other substances than ether, as determined by weighing the residue after evaporation.

Mixtures of alcohol and ether in equal parts did not serve better than alcohol alone for the extraction of wet and dried tissues.

Ethereal extracts therefore, cannot be utilized for antigen because of their highly hemolytic properties; however, they usually contain large amounts of antigenic substances, principally lecithin, that may be removed from the crude ether extracts and utilized for antigen by precipitation with an excess of acetone.

Alcohol versus Acetone Extraction.—Kolle and Steiner[26] have advocated the extraction of dried tissues with acetone for antigen in the Wassermann test but Laubaugh, Gasselman, Williams and myself have found acetone extracts of syphilitic liver somewhat inferior to alcoholic extracts.

In the study by Miss Trist and myself plain acetone extracts of wet and dried non-syphilitic human heart and liver and beef heart usually showed antigenic activity but somewhat less than extracts prepared with absolute ethyl alcohol under identical conditions; the anticomplementary and hemolytic activities of both kinds of extracts were usually quite similar.

Ninety-five Per Cent versus Absolute Ethyl Alcohol.—Absolute ethyl alcohol is commonly used for the extraction of tissues, but owing to the cost of high grade alcohol it is a matter of practical importance to ascertain if the usual 94 to 95 per cent may be utilized for the extractions of tissues.

The results of experiments by Trist and myself employing fresh and dried human and beef heart muscle, have shown that extracts made with 95 per cent alcohol are quite similar in all properties to those made with "absolute" ethyl alcohol; the latter extracts were however, occasionally slightly superior in antigenic activity; and for this reason *"absolute" ethyl alcohol is to be preferred and especially for the extraction of wet tissues, but is not absolutely necessary for the preparation of extracts.*

Ethyl versus Methyl and Isopropyl Alcohols.—Custom has sanctioned the use of ethyl alcohol for the extraction of tissues, but numerous experiments by Trist and myself have shown that almost as good extracts are obtained with methyl alcohol providing dried tissues are employed. Curiously, methyl alcohol extracts of wet tissues, as fresh minced beef heart, were usually decidedly inferior in antigenic activity to ethyl alcohol extracts prepared at the same time and in the same manner. Our experiments were conducted with high purity absolute ethyl alcohol and acetone-free methyl alcohol of highest purity obtainable. No differences were observed in an anticomplementary and hemolytic activities of extracts prepared with these alcohols. Either alcohol may be used successfully for the extraction of dried tissues but ethyl alcohol is better for the extraction of wet or fresh tissues.

Isopropyl alcohol has been advocated by Isham[27] because of its cheapness but antigens prepared of desiccated beef heart by Miss Yagle and myself were found to be less antigenic than extracts employing ethyl alcohol while the hemolytic and anticomplementary properties were the same. Furthermore comparative Wassermann tests showed that the isopropyl extracts gave weaker reactions and not unfrequently falsely negative reactions.

Temperature in Relation to Extraction of Tissues with Alcohol.—Tissues are generally extracted in an incubator at 38° C. Comparative experiments consisting of extraction of non-syphilitic human heart and liver and beef heart with alcohol at 38° C. (incubator), 22° C. (room) and 6° C. (refrigerator) have shown that all are satisfactory.

Similar results have been observed with extracts of human heart and liver and indicate that *extraction of tissues with alcohol at temperatures varying from 6° to 38° C. yield antigens of similar properties; usually, however, extractions at 38° C. guarding against evaporation and adding alcohol to make up for the inevitable slight loss, have yielded extracts slightly superior in antigenic activity in a somewhat shorter*

period of time and for these reasons this temperature is to be preferred, but is not absolutely necessary for the preparation of good extracts.

Ecker and Sasano[28] have described a method for the preparation of extracts in one to three hours with boiling alcohol; Miss Trist and I have tried this method with wet and dried beef heart before and after preliminary extraction with ether in a Sohxlet apparatus, but the extracts were distinctly less antigenic than extracts prepared at the same time by more prolonged extraction in an incubator.

Naturally I have been much interested in the possibility of preparing my antigen in a short period of time by using boiling ether and alcohol. When extracts were prepared by powdered beef heart in the usual manner described in Chapter XXXI by extracting at room temperature with ether for five days followed by 95 per cent alcohol in an incubator for four days and lastly with absolute ethyl alcohol for six days, the acetone-insoluble lipoids from the ether and primary alcoholic extract being returned to the secondary alcoholic extract along with 0.2 per cent cholesterol, the results of titrations by my method have been usually as follows:

Not hemolytic in 0.5 cc. of 1 to 4
Anticomplementary in 0.5 cc. of 1 to 6
Antigenic in 0.5 cc. of 1 to 3200

Now when an extract of the same tissue was prepared at the same time and in the same manner except that the ether extraction was for two hours boiling and the primary and secondary alcohol extractions for two hours boiling, the results of titrations have been usually as follows:

Not hemolytic in 0.5 cc. of 1 to 4
Anticomplementary in 0.5 cc. of 1 to 6
Antigenic in 0.5 cc. of 1 to 1200 to 1 to 3200.

In other words the shorter method employing boiling alcohols may sometimes yield extracts of lesser antigenic sensitiveness, but on the whole it is serviceable and satisfactory and the details of preparation are given in Chapter XXXI.

Time Required for the Extraction of Tissues with Alcohol.—Tissues are usually extracted with alcohol at 38° C. for four to fourteen days, although some authors have advocated twelve hours and others several weeks.

In our experience hemolytic, anticomplementary and antigenic principles may be present in the alcohol on and after the first day of extraction, *but the extracts do not usually reach their maximum in antigenic activity until the fifth to eighth days;* from the first to eighth days the extracts acquire slightly greater hemolytic and anticomplementary activity *but especially gain in antigenic activity.* For these reasons we believe the extraction of wet and dried tissue with

alcohol at 38° C. should be seven to eight days at least, nothing being gained by more prolonged extractions.

Alcoholic Extraction of Dried versus Wet Tissues.—Dried tissues and particularly fetal syphilitic liver and beef heart muscle, have been used by a few workers for many years for the purpose of extraction with acetone or alcohol.

The principal advantages are the *finely divided state* of the material, facilitating the extraction of intracellular substances and particularly phosphatids (Erlandsen), and *convenience.* Large amounts of muscle may be ground and dried at one time and readily kept in glass-stoppered bottles. The method also facilitates the *use of mixtures* of muscle from different hearts, which has a distinct advantage over the use of the muscle of one heart for the preparation of an extract.

The main disadvantage is that alcoholic extracts of dried muscle are quantitatively *more hemolytic* than extracts of wet tissue; the extraction of dried muscle with ether however, by my method usually removes all of these hemolytic substances. Rapid drying in a vacuum apparatus yields a product less hemolytic than material slowly dried at room temperature; for this reason I believe that autolytic changes facilitated by slow drying result in the production of these hemolytic substances. Dried heart muscle therefore has some advantages over wet tissue and for several years I have used that prepared by the Digestive Ferments Company of Detroit with complete satisfaction.

The Amount of Tissue Extracted.—The proper amount of wet or dried human or beef heart tissue to employ for alcoholic extraction would appear to be from 4 to 10 gms. per 100 cc. of alcohol; less tissue yields an extract of comparatively weak antigenic activity. The use of more than 10 gms. of tissue results in increasing the hemolytic and anticomplementary activities of some extracts without an appreciable gain in antigenic activity; I have used 10 gms. of ether extracted powder for each 100 cc. of 95 per cent or absolute alcohol with success and general satisfaction.

The Influence of Reënforcing Tissue Extracts with Cholesterol.—While alcoholic solutions of cholesterol are at best feebly antigenic, undoubtedly the addition of cholesterol to alcoholic extracts of tissues greatly improves antigenic activity. As far as I know all serologists agree with this statement, but a few are of the opinion that such cholesterol reënforced extracts may yield non-specific reactions.

The amount of cholesterol used by different workers varies rather widely; for example, Browning and McKenzie add 1 or 1.2 gms. to each 100 cc. of their 0.75 per cent solutions of liver lecithins in ethyl alcohol; Sachs uses 0.1 per cent or less added to alcoholic extracts of heart; Walker and Swift regard 0.4 per cent as optimum for alcoholic

extracts of heart and the majority of serologists in this country have apparently adopted this practice. Field uses alcoholic extracts of heart muscle one-half saturated with cholesterol; Neymann and Gager use 0.2 per cent solutions in alcoholic extracts of heart. Cholesterol being antihemolytic usually increases the anticomplementary activity of tissue extracts; experiments by Trist and myself have sought to determine the minimal amount necessary to *stabilize* extracts and enhance their antigenic activity without greatly increasing anticomplementary activity. With this end in view a large number of antigens were prepared and titrated the results of which may be summarized as follows:

1. Solutions of cholesterol in plain ethyl alcohol are more anticomplementary than in alcoholic extracts of heart due presumably to the presence of hemolytic substances in the latter.

2. *The addition of 0.1 to 0.2 gm. pure cholesterol to 100 cc. alcoholic extracts of heart is sufficient to stabilize the antigen and render it highly antigenic.* Occasionally the addition of more cholesterol up to 0.4 per cent results in enhancing antigenic activity to a slightly greater degree, but this also increased anticomplementary activity and accordingly the chances for non-specific reactions. The results of these and numerous other experiments of a similar character have shown that *the addition of 0.1 or 0.2 gm. pure cholesterol to 100 cc. alcoholic extracts of beef or human heart imparts the maximum or almost the maximum of antigenic activity and, in my opinion, constitutes sufficient for gaining all the advantages to be derived from cholesterolizing tissue extracts.*

Influence of Adding Formalin to Antigen.—Reyner[29] has recently stated that adding Merck's formalin to my antigen diluted with saline to give a final concentration of 1 to 1000 (for example 0.1 cc. of formalin to 99.9 cc. of 1 to 300 of antigen) increased the rate and degree of complement-fixation in syphilis without danger of producing non-specific reactions. Miss Yagle and I however, have found that formalized antigen produces many non-specific reactions; in one series of comparative tests with plain antigen almost 40 per cent of non-syphilitic sera gave weak fixations with the 0.1 and 0.05 cc. amounts of serum after primary incubations of four and eighteen hours at 6° to 8° C. Therefore I regret being unable to endorse the method although it does increase slightly the degree of fixation by syphilitic serum as one would expect under these circumstances. Dold[30] had previously found that 1 to 300 formalin was destructive for complement and hemolysin but while 1 to 1000 is but slightly destructive for complement (unit of 1 to 30 titrated in presence of plain antigen 0.3 cc.; unit in presence of formalized antigen 0.45 cc.), it tends to interfere with hemolysis and to produce weak non-specific reactions. Forssman[31] states that large amounts of formalin are destructive for all antibodies except the antitoxins including those concerned in the Wassermann and Sachs-Georgi reactions.

COMPARATIVE ANTIGENIC, ANTICOMPLEMENTARY AND HEMOLYTIC ACTIVITIES OF EXTRACT ANTIGENS.

Comparative Antigenic Activities.—The following four methods are available for determining the comparative antigenic activity of tissue extracts:

1. By using each extract in varying amounts with constant amounts of syphilitic serum and complement to determine the antigenic unit or smallest amount of antigen giving complete inhibition of hemolysis.

2. By using each extract in constant amount of 2 or 5 antigenic units as determined in 1 with a constant amount of complement and varying amounts of syphilitic serum.

3. By using each extract in constant amount of 2 or 5 antigenic units with a constant amount of syphilitic serum and a varying number of units of complement.

4. By routine Wassermann tests with a large number of sera.

Miss Trist and I have employed the first three methods in comparative studies of a large number of different extracts. While the fourth method is the one generally employed, it is subject to error in mistaken clinical diagnoses, whereas with the first three methods only sera from absolutely known cases of syphilis are employed.

Furthermore, the method of primary incubation influences the results, that is, cold incubation increases the antigenic activity of all extracts, but especially of the plain or crude alcoholic extracts; in our experiments both warm (water-bath, one hour) and cold (refrigerator, eighteen hours) incubation were employed.

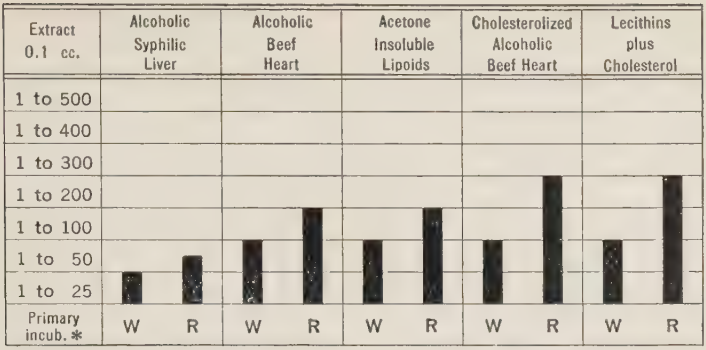
All experiments were conducted with *mixtures* of fresh sera from 6 or more syphilitic individuals; likewise mixed guinea-pig serum complements were employed with an antisheep hemolytic system, the details of technique being the same as described in Chapter XXXI.

As previously stated the extracts in common use are plain or crude alcoholic extracts of heart (human, beef or guinea-pig), cholesterolized alcoholic extracts of heart and acetone-insoluble lipoids of heart or liver; to these may be added a fourth, namely, an extract of alcoholic solutions of lecithins plus cholesterol (Browning and McKenzie; Neymann and Gager).

The results of an experiment with four of these extracts tested for antigenic activity according to the first method in a water-bath at 38° C. for one hour and in a refrigerator at 8° C. for eighteen hours are shown in Fig. 3. Fig. 4 gives the results of an experiment conducted after the second method and Fig. 5 with the third method.

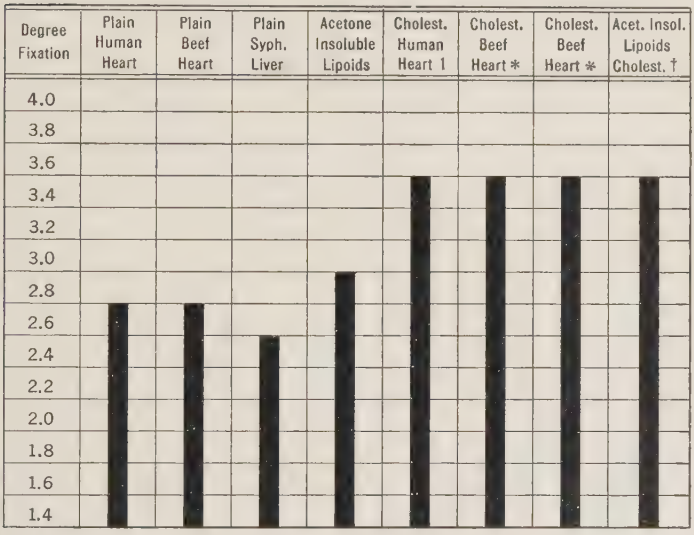
The general results may be summarized as follows:

1. Cholesterolized extracts are most antigenic at both 38° C. and



* W = water-bath one hour; R = refrigerator 8° C. for eighteen hours.

FIG. 3.—Comparative antigenic activity of tissue extracts.



* 0.4 per cent cholesterol. † 0.2 per cent cholesterol.

FIG. 4.—Comparative antigenic activity of extracts at 38° C. for one hour.

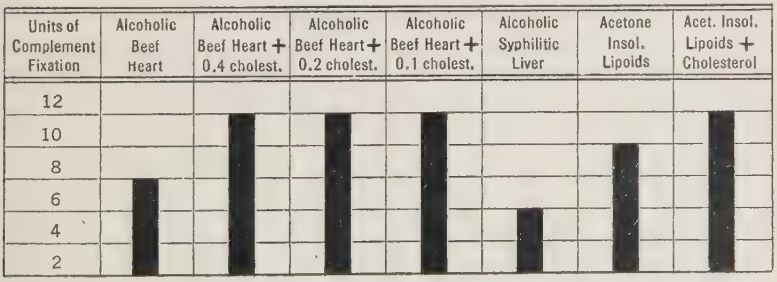


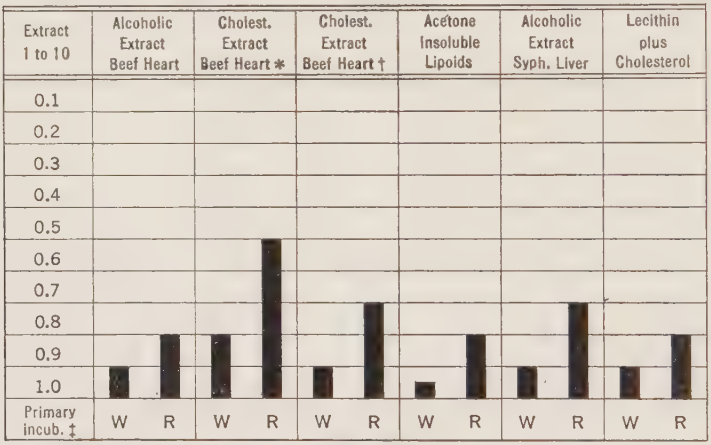
FIG. 5.—Comparative antigenic activity of organ extracts at 8° C. for eighteen hours.

8° C.; next in order of sensitiveness are acetone-insoluble lipoids and plain alcoholic extracts at both temperatures.

2. Extracts containing 0.2 per cent cholesterol are usually, but not always, as antigenic as those containing 0.4 per cent.

3. Mixtures of lecithins (acetone-insoluble lipoids) and cholesterol are highly antigenic and usually as much so as alcoholic extracts of heart reënforced with 0.2 to 0.4 per cent cholesterin.

Comparative Anticomplementary Activities.—In all experiments an antisheep hemolytic system was employed and the results of one experiment are shown in Fig. 6. Extracts of acetone-insoluble lipoids were least anticomplementary followed in order by acetone-insoluble lipoids plus cholesterol, plain alcoholic extracts of heart and syphilitic liver and by 0.2 to 0.4 per cent cholesterol reënforced extracts.



* 0.4 per cent cholesterol. † 0.2 per cent cholesterol.

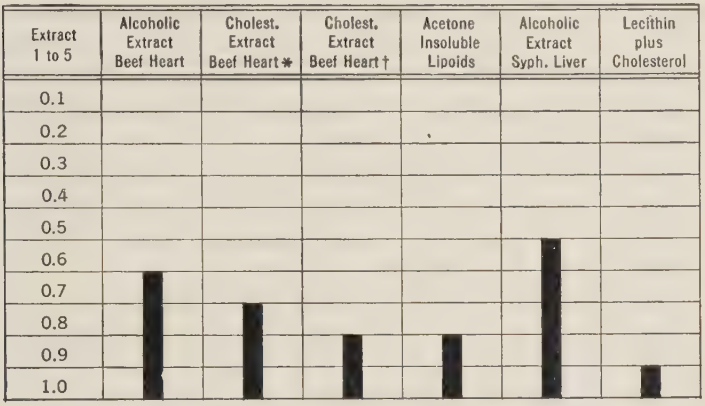
‡ W = water-bath one hour; R = refrigerator eighteen hours.

FIG. 6.—The anticomplementary activity of tissue extracts at 38° C. for one hour and at 8° C. for eighteen hours.

Comparative Hemolytic Activities.—Various substances in alcoholic extracts of tissues are regarded as possessing hemolytic properties; chief among these are the soaps and fatty acids.

Hemolytic activity of extracts of different tissues of the same kind and prepared in exactly the same manner is known to vary, but the object of our experiments was to test a sufficient number representing the four main kinds to permit of broad comparison and conclusions. The results of an experiment shown in Fig. 7, are representative of all tests of this kind and show the comparative hemolytic activities of different extracts for 0.5 cc. of 2 per cent suspensions of sheep cells in a total volume of 2 cc. and an exposure of one hour at 38° C. in a water-bath.

These experiments have shown that extracts of acetone-insoluble lipoids (lecithins) and mixtures of these with cholesterol are least hemolytic; next in order are cholesterolized alcoholic extracts of heart, plain alcoholic extracts of heart and alcoholic extracts of fetal liver, the latter being usually most hemolytic due, probably, to the presence of autolytic products in addition to a high content of fatty acids and soaps.



* 0.4 per cent cholesterol.
† 0.2 per cent cholesterol.

FIG. 7.—The hemolytic activity of tissue extracts at 38° C. for one hour.

PRINCIPLES OF THE AUTHOR'S METHOD FOR PREPARING ANTIGEN.

The following method[32] utilizes these observations and yields extracts of remarkable antigenic sensitiveness and slight anticomplementary and hemolytic activity; the principles of the method are as follows the details of technique of preparation being given in Chapter XXXI.

1. Dried human or beef heart muscle is employed; there is no choice between the two tissues if both are *perfectly fresh*. Human heart muscle, however, is frequently fatty or may have undergone more or less postmortem change; if so, fresh lean beef heart muscle is to be preferred. Dried tissue is employed for the following reasons: (*a*) It permits of the use of very finely divided tissue; (*b*) tissues are easily kept for long periods of time; (*c*) three or more heart powders may be kept, permitting of the use of mixtures of tissues and polytropic extracts which are probably better than extracts prepared of a single heart muscle.

2. The dried tissue is first extracted with ether, which removes a large portion of the hemolytic substances; the phosphatids (lecithins)

which are also partly removed by the ether are recovered by precipitation with acetone and returned to the extract.

3. The tissue is now dried and extracted with alcohol which removes some antigenic and hemolytic substances and considerable anticomplementary substances. This alcohol is evaporated, extracted with ether and precipitated with acetone, which removes the highly antigenic fraction of lecithins (phosphatids), which are later returned to the extract.

4. The tissue is now extracted for a second time with absolute ethyl alcohol which removes some antigenic principles and slight or almost negligible amounts of anticomplementary and hemolytic substances. The acetone-insoluble lipoids (lecithins, phosphatids) recovered from the preliminary ether and alcohol extractions are dissolved in ether and returned to this alcoholic extract with 0.2 per cent cholesterol. The resulting extract posseses high antigenic activity, slight anticomplementary and frequently no hemolytic activity as shown in the results of titrations as follows:

1. Primary ether extract:	
Hemolytic	0.5 cc. of 1 to 8
Anticomplementary	0.5 cc. of 1 to 4
Antigenic	0.5 cc. of 1 to 50
2. Primary alcohol extract:	
Hemolytic	0.5 cc. of 1 to 4
Anticomplementary	0.5 cc. of 1 to 16
Antigenic	0.5 cc. of 1 to 20
3. Secondary alcohol extract:	
Hemolytic (not in)	0.5 cc. of 1 to 2
Anticomplementary	0.5 cc. of 1 to 10
Antigenic	0.5 cc. of 1 to 125
4. Finished extract:	
Hemolytic (not in)	0.5 cc. of 1 to 2
Anticomplementary	0.5 cc. of 1 to 8
Antigenic	0.5 cc. of 1 to 2400

The above results indicate the following: 1. The primary ether extraction removes considerable of the hemolytic and some of the anticomplementary and antigenic principles.

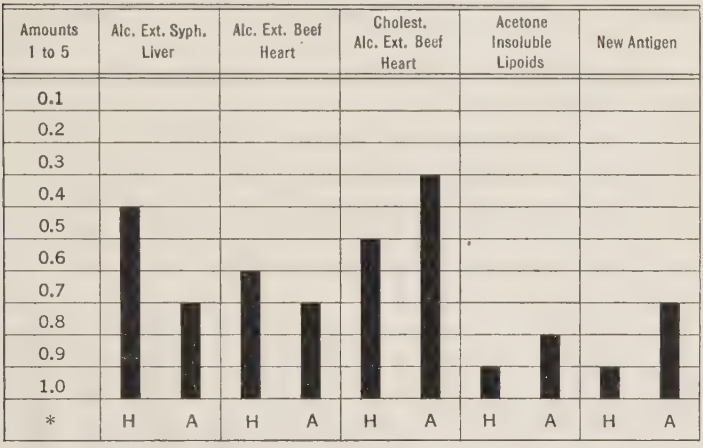
2. The primary alcoholic extraction also removes some of the hemolytic, anticomplementary and antigenic substances.

3. The secondary alcoholic extract is much less hemolytic and anticomplementary than the primary alcoholic extract while being equal in antigenic activity (sometimes greater).

4. The secondary alcoholic extract reënforced with the acetone-insoluble lipoids (lecithins; phosphatids) and 0.2 per cent cholesterol is not any more hemolytic but is slightly more anticomplementary (due to cholesterol) than the alcoholic extract alone; its antigenic activity is greatly increased, the antigenic unit being one hundred to

three hundred times less than the anticomplementary and hemolytic units.

The finished extract easily permits the use of at least 10 antigenic units as the dose to be employed in the conduct of complement-fixation tests, this amount being at least thirty times less than the anticomplementary and hemolytic units and rendering the test for syphilis highly sensitive without the danger of non-specific reactions.



* H = hemolytic unit: A = anticomplementary unit.

FIG. 8.—Comparative hemolytic and anticomplementary activities of various antigens.

Comparative Value of the Author's Antigen.—A study of the properties of the new antigen has been made in a series of comparative tests employing the usual antigens of plain and cholesterolized alcoholic extracts of heart muscle, alcoholic extracts of fetal syphilitic liver, and acetone-insoluble lipoids of heart muscle (3 per cent solutions in methyl alcohol as prepared by Noguchi).

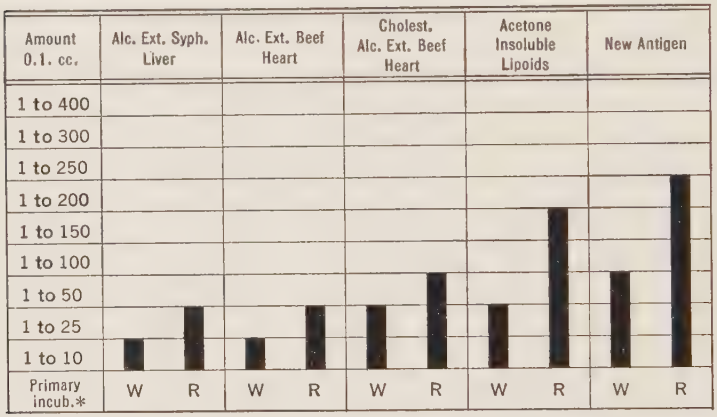
The results of one experiment are shown in Figs. 8 and 9 as examples of the series; Fig. 8 gives the comparative hemolytic and anticomplementary values and Fig. 9 the comparative antigenic values.

As shown in these charts the new antigen has been found superior to those commonly employed, being very highly antigenic and remarkably free of the objectionable hemolytic and anticomplementary properties.

In these respects it approaches the requirements of an ideal extract somewhat more closely than the extracts in common use; furthermore, being prepared of a mixture of dried muscles from several hearts and containing but 0.2 per cent cholesterol, it is highly poly-

tropic and has never yielded negative complement-fixation reactions, when tested with a series of antigens yielding positive reactions with the sera of syphilitic persons. For this reason *the new antigen may be safely relied upon alone and the necessity for using multiple antigens in the conduct of the Wassermann test for the sake of accuracy is thereby removed.*

The new antigen is not as cheaply prepared as simpler extracts owing to the alcohol, ether and acetone employed, but when considered in view of the large amount of finished extract (100 cc.) and the very small amount necessary for a complement-fixation test, the expense is relatively small.



* W = water-bath one hour; R = refrigerator at 8° C. for eighteen hours.

FIG. 9.—Comparative antigenic values of various antigens.

The technique of preparation is simple although more laborious than the preparation of simple alcoholic extracts; it is scarcely more laborious however, than the preparation of acetone-insoluble lipoids as described by Noguchi and Bronfenbrenner.

THE PRESERVATION OF ANTIGEN.

The new antigen keeps remarkably well either in a refrigerator or at room temperature. As shown by Kilduffe[33] it is quite stable and withstands extremes of temperature and long storage (two to three years) with relatively little change. Furthermore, as reported by the same serologist,[34] the desiccated and powdered beef heart "is quite stable, satisfactory antigens having been prepared from it after eleven months. No special precautions in keeping the powder are required other than to keep the containers tightly stoppered to prevent the absorption of moisture. If the precaution is taken to

shake the container *frequently* and vigorously during extraction, antigens of remarkable potency may be prepared; without special care, the extracts are highly antigenic and quite satisfactory."

Similar results have been observed by Miss Yagle and myself and in a special study of the preservation of this antigen as well as of numerous other extracts both in alcoholic solution and after dilution with saline solution the following conclusions were reached:[34]

1. Various alcoholic extracts of tissues preserved in an incubator at 37° C. and in a laboratory at 22° C. tend to lose slightly in antigenic activity, as compared with portions kept in a refrigerator at 6° to 8° C.

2. Practically no changes in the anticomplementary and hemolytic activities of various alcoholic extracts of tissues takes place over a period of twenty-eight days in portions kept in an incubator at 37° C., in a room at 22° C. and in a refrigerator at 6° to 8° C. Plain alcoholic extracts of heart, however, sometimes acquire slightly greater hemolytic activity when preserved at 37° C.

3. Emulsions of various alcoholic extracts of tissues prepared with physiological saline solution and kept in a refrigerator at 8° C., do not undergo any marked or constant changes in antigenic activity over a period of twenty-eight days.

4. The preserved emulsions of each extract, however, tend to become more anticomplementary and slightly more hemolytic, as compared with freshly prepared emulsions, due largely to bacterial contamination. For this reason we believe *it is well to dilute only enough antigen with saline solution for the tests to be done each day* although a saline preparation may be carried over in the refrigerator for several days with satisfactory results.

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CHAPTER XV.

ANTIGENS FOR BACTERIAL COMPLEMENT-FIXATION TESTS WITH SPECIAL REFERENCE TO THE KOLMER METHODS OF PREPARATION.

A LARGE number of methods have been advocated for the preparation of bacterial antigens and the subject is one of primary importance in relation to the sensitiveness and practical value of complement-fixation in bacterial infections.

The primary object in the preparation of any antigen for complement-fixation work is to secure one with a long range or the *maximum antigenic index*, that is, an antigen possessing a high degree of antigenic sensitiveness with the minimum of anticomplementary activity. All antigens actually containing some bacterial substance possess some anticomplementary activity or the power for non-specific fixation of complement, but not all possess antigenic activity or the power for specific fixation complement with specific antibody. This fact complicates complement-fixation with bacterial antigens because the use of one-half, one-third or one-fourth the anticomplementary unit of an antigen by no means insures the use of an antigenic dose unless this has been determined by an additional antigenic titration or unless the antigen is prepared by a method known to result in antigenic sensitiveness.

THE CHEMISTRY OF BACTERIAL ANTIGENS.

The substances in bacteria capable of acting as true antigens in the sense of stimulating the production of antibodies may not be identical with those acting as antigens in the test-tube in complement-fixation reactions. Indeed available evidence indicates that the lipoids of bacteria and protozoa and notably the acetone-insoluble lipoids (lecithins) are active antigens in the test-tube while protein-free extracts of these lipoids are unable to produce antibodies in animals.

In all probability the proteins or protein-lipoid complexes of bacteria, molds and protozoa are the active antigens in the living animal in the production of antibodies but what is the chemical nature of the components acting as antigens in the test-tube in the fixation of complement? Hitherto most immunologists have considered the proteins as the active antigens. This view has been

based largely upon the fact that alcoholic, ether, chloroform, acetone, carbon tetrachloride and other lipoid-dissolving extracts of bacteria have frequently failed as antigens in complement-fixation tests although many observers have employed alcoholic extracts of tubercle bacilli with success.

But failure with extracts of lipoid solvents does not necessarily indicate that the lipoids are without antigenic activity in the test-tube in complement-fixation; it may be largely a matter of quantity present and their colloidal state. Probably a purification and concentration of the lipoids would yield different results and indeed this is indicated by the work of Warden[1] with the fatty acids of various bacteria, of Patterson and Williams[2] and Fairley[3] with the acetone-insoluble lipoids of hydatids and bilharzia and more recently by Wadsworth and the Maltaners[4] and others who found the acetone-insoluble lipoids of tubercle bacilli highly antigenic. Wassermann[5] has also stated that the addition of lecithin to the specific proteins of the tubercle bacillus increases antigenic activity.

A rather large literature has accumulated upon the subject and especially in relation to the tuberculosis complement-fixation test with the general result that increasing attention is being commanded by the lipoids of bacteria, molds and protozoa in relation to the complement-fixation reaction. In a way this is in conformity with our knowledge of the tissue lipoids and especially the lecithins as antigens for the Wassermann reaction although while these are non-specific it would appear that the bacterial and protozoal lipoids are highly specific and that tissue lipoids cannot be substituted for them in complement-fixation tests. On the other hand the high specificity of the bacterial lipoids in complement-fixation suggests that they are not pure lipoids but rather lipoids in combination with specific proteins.

Indeed there can be little or no doubt that the proteins of bacteria act as antigens in the complement-fixation reaction although it may be, as just stated, that these are not pure proteins but protein-lipoid complexes. Furthermore, in the preparation of antigens, especially of the tubercle bacillus, it would appear advisable to remove some of the lipoids at least in order to reduce anticomplementary activity and *especially to remove the chances of falsely positive reactions with syphilis antibody,* to which further reference will be shortly made. Certainly ordinary alcoholic, acetone, ether and chloroform extracts or suspensions of bacteria are apt to fail as antigens in complement-fixation tests and are far inferior to watery or saline extracts containing the proteins and probably lipoids or protein-lipoid complexes in a colloidally dispersed state. Insofar as the tubercle bacillus is concerned Dienes and Schoenheit,[6] while recognizing the antigenic importance of constituents extractable by lipoid solvents, stress the fact that with their sera antigens

residing with the protein fractions are also demonstrable and that some sera react with the lipoidal antigens and others with the protein. But it is possible that their protein antigens contained traces of lipoidal substances. Even a carbohydrate fraction of tubercle bacilli has been found by Laidlaw and Dudley[7] and by Dienes and Schoenheit[8] to possess antigenic activity and is possibly similar to those obtained from the pneumococcus by Avery and Heidelberger, but the possibility remains that this substance may be found associated with either the proteins or the lipoidal fractions of tubercle bacilli according to the technique of preparation.

Just what part of the bacterial cell is most antigenic in complement-fixation reactions is difficult to state. Doubtless the ectoplasm is important because I have found that antigens prepared by suspending typhoid bacilli and other organisms from agar cultures in saline solution and centrifuging at once yield supernatant fluids of antigenic activity just as Perlzweig and Keefer[9] and Ferry and Fisher[10] have noted the high antigenic activity of such ecto-antigen in the production of antibodies in the lower animals. Hofmeier[11] has also found the antibody for the ectoplasm of the typhoid bacillus different from that for the endoplasm and believes that complement-fixation with antigens of whole typhoid bacilli is largely due to the presence of ectoplasmic constituents.

IMPORTANCE OF THE METHOD EMPLOYED IN THE PREPARATION OF BACTERIAL ANTIGENS.

In some bacterial infections notably typhoid fever and glanders, relatively large amounts of complement-fixing antibody are usually present in the serum, rendering complement-fixation reactions highly satisfactory and minimizing the importance of the method employed for the preparation of antigen; in these diseases the antigen may be prepared by any recognized method and is likely to prove satisfactory. But in other bacterial infections notably gonorrhea and tuberculosis, the amount of antibody in the serum is likely to be small and the method employed in the preparation of antigen exerts a very important influence upon complement-fixation reactions and is a subject of first rate importance in relation to complement-fixation tests for these infections. The large number of methods described and to be found in the literature attests to the unsatisfactory status of this subject and *it is highly probable that no one method is applicable to all bacteria.*

A CLASSIFICATION OF BACTERIAL ANTIGENS.

Bacterial antigens may range from simple suspensions or extracts of whole bacteria in saline solution or alcohol to very complex anti-

gens of partly or completely disintegrated bacteria with or without their secretory products and constituents of the culture medium. Miller[12] has divided antigens of the tubercle bacillus into four groups as follows: Group I, antigens composed of whole bacilli; Group II, antigens consisting of the tuberculins; Group III, antigens which may be designated as "tubercle bacillus derivations or extracts;" and Group IV, antigens produced from normal and from tuberculous tissues.

With bacterial antigens in general a larger classification is necessary to differentiate, for example, suspensions in fluid media from suspensions of washed bacteria in saline and both of these as to whether the suspensions are used living or after being killed by heat or a chemical, as phenol or tricresol. Furthermore, centrifuging and filtration through a substance capable of holding back the whole or partially disintegrated bacteria alter the properties of antigens. Likewise, whether or not the bacterial fats are allowed to remain or are partially or entirely removed and the protein residue or protein-lipoid complexes used as antigen, etc.

The earliest bacterial antigens used by Bordet and Gengou[13] were simple saline suspensions while Wassermann and Bruck[14] employed autolysates in distilled water. Gradually more complicated methods were introduced and especially for the preparation of antigens of the tubercle bacillus, until as many as twenty-five more or less distinctive methods are known for the preparation of bacterial antigens, which I have classified into seven groups as follows:

Group I.—*Suspensions in fluid culture medium* of which there are three kinds: (*a*) living; (*b*) killed by heat and (*c*) killed by a chemical germicide. Such antigens contain whole microörganisms, their secretions into the culture medium, probably slight amounts of autolytic products and the constituents of the medium. They may be modified by heat or a chemical germicide and hence the necessity for subdivisions.

Group II.—*Suspensions of washed or unwashed bacteria in saline solution* of which there are three kinds: (*a*) living; (*b*) killed by heat and (*c*) killed by a chemical germicide. These antigens differ from Group I antigens in being free of the constituents of the culture medium and autolytic products of the bacteria, although the latter are added when the antigens are kept for two weeks or longer.

Group III.—*Autolyzed bacteria in water.* Autolysis may be aided by freezing and thawing, heating, or by the addition of alkalies (antiformin). These antigens are solutions of the bacterial cells and their contents. Sometimes they are centrifuged and the supernatant fluids (centrifugates) employed.

Group IV.—*Bacteria disintegrated by drying and grinding into powder,* including the dried and disintegrated precipitates secured with alcohol. These antigens contain the soluble constituents of bacteria plus the finely divided insoluble portions in suspension.

Group V.—*Filtrates* of broth cultures (Koch's old tuberculin and mallein) including filtrates of cultures autolyzed in water and filtrates of solution suspensions of dried and pulverized bacteria.

GROUP VI.—*Lipoid extracted bacteria.* In these antigens the fats of whole or dried and pulverized bacteria are removed partially or completely by such solvents as alcohol, ether, chloroform, carbon tetrachloride, etc. The remaining nucleoproteins or protein-lipoid complexes constitute the main constituents of the antigens.

GROUP VII.—*Solutions or emulsions of the bacterial fats.* Typical antigens of this class may be free of protein as prepared by Warden; ordinarily they are protein-lipoid complexes in the form of acetone-insoluble lipoids (mostly lecithins), etc.

METHODS COMMONLY EMPLOYED FOR THE PREPARATION OF BACTERIAL ANTIGENS.

The following methods classified under the above groups do not by any means include all that have been described by various investigators, but were selected as representative methods based upon more or less distinctive principles and employed for a series of studies with tubercle bacilli, typhoid bacilli, glanders bacilli and gonococci, upon this subject of bacterial antigens.

Preparation of Group I Antigens.—*Method* 1.—Twenty-four to forty-eight hour (longer for tubercle bacilli) growths in neutral broth, briefly shaken to break up clumps and used at once in a living state.

Method 2.—Same as Method No. 1 except the culture is heated at 60° C. for one hour or autoclaved for sterilization and preserved with 0.5 per cent phenol.

Method 3.—Same as Method No. 1 except the culture is unheated but sterilized and preserved with 0.5 per cent phenol or 0.5 per cent phenol and 10 per cent glycerin.

Preparation of Group II Antigens.—*Method* 4.—Young cultures are removed from solid media with saline solution and washed, resuspended in saline, briefly shaken to break up clumps and used at once in a living state.

Method 5.—Young cultures are removed from solid media with saline solution and centrifuged; or broth cultures are centrifuged. The sediment of bacteria is washed once or twice with saline solution, suspended in saline, shaken to break up clumps, heated at 60° C. for one hour and preserved with 0.5 per cent phenol.

Method 6.—Same as Method No. 5 except the suspensions are not heated but sterilized and preserved with 0.5 phenol or 0.5 per cent phenol and 10 per cent glycerin.

Method 7.—Young cultures are removed from a solid medium with saline solution and centrifuged or filtered through paper. The partially dried bacterial residue is weighed and gradually but thoroughly rubbed up in a mortar for three or four hours with sufficient saline solution to yield a 0.5 per cent suspension, sterilized and preserved by adding one-tenth volume of 5 per cent solution of phenol. This is a brief description of the Fleisher-Ives method for preparing an antigen of tubercle bacilli.[15]

Method 8.—Young cultures are removed from a solid medium with saline solution and centrifuged (living or after heating the suspension at 60° C. for one-half hour). To each 0.02 gm. of bacterial residue in a centrifuge tube is added 0.09 gm. of sodium chloride followed by stirring with a glass rod for one hour when 10 cc. of water are added. This is the Miller-Zinsser method for preparing an antigen of tubercle bacilli.

Preparation of Group III Antigens.—*Method* 9.—Young cultures are washed from solid media with sterile distilled water or are washed from solid media with saline, centrifuged and the sediment suspended in sterile water or secured from broth by centrifuging and suspending the sediment in water. These are shaken for two days at room temperature, sterilized and preserved with 0.5 per cent phenol. This is the Wassermann-Bruck method of preparing artificial aggressins, an antigen used for complement-fixation in typhoid fever.

Method 10.—Same as the preceding method except that the emulsions are heated at 60° C. for twenty-four hours, shaken for two days and preserved with 0.5 per cent phenol (Leuch's[16] antigen).

Method 11.—Same as the preceding except that the emulsion is heated at 60° C. for one hour, shaken for twenty-four hours, frozen hard for an hour and thawed on each of three days successively.

Method 12.—The water-soluble partial antigen of tubercle bacilli of Deyke and Much.[17] A 5 per cent suspension of well-washed tubercle bacilli is made up in a 1 per cent solution of lactic acid in distilled water and completely disintegrated at 58° C. for five weeks with occasional periods of shaking, followed by paper filtration. The filtrate (A) is used and is said by Deyke and Much to contain very small amounts of dissolved protein, a minute amount of lipoids and to closely resemble tuberculin in its action.

The residue is subject to extractions with alcohol at varying temperatures for the fatty acids and other lipoids and these "partial antigens" are listed and described under Group IV antigens.

Method 13.—Bacteria are removed from solid media with sterile water or secured from broth cultures by centrifuging and resuspended in sterile water. This bacterial suspension is heated at 60° C. for one hour and shaken for several hours followed by freezing in an ice and salt mixture for one hour. It is then permitted to thaw at room temperature followed by a second freezing and thawing the same day and shaken over night. The following day the freezing and thawing are repeated twice more followed by shaking over night. Next day the emulsion is thoroughly centrifuged and the supernatant fluid used as antigen preserved with 0.5 phenol. This method is that described by Dean[18] for the preparation of antigen of the bacilli of the typhoid-colon group.

Method 14.—Washed bacteria are suspended in 5 cc. of saline solution to which is added 5 cc. of absolute ethyl alcohol. The precipitate is secured by centrifuging, dried in a vacuum, ground to a fine powder and each 0.01 gm. placed in 5 cc. of a 2 per cent solution of antiformin in water. This mixture is heated in a water-bath to 56° C. until the bacteria are dissolved. The solution is neutralized with decinormal sulphuric acid (litmus indication) and 1 or 2 drops of 5 per cent sodium thiosulphate are added and the end results tested with potassium iodide starch paper. The solution is now made up to 10 cc. with phenolized saline solution and centrifugalized. The supernatant fluid is used as antigen, each cubic centimeter containing the extract of 0.001 gm. of the dried ground precipitate. This method was described by Kinsella and Swift[19] for the preparation of antigens of streptococci.

Method 15.—Bacteria are washed and prepared in a heavy suspension in saline solution; antiformin is added to give a concentration of 5 per cent and the mixture boiled for several minutes to dissolve the bacteria and until the emulsion is translucent. The solution is now neutralized (to litmus) with normal hydrochloric acid, treated with several volumes of 95 per cent alcohol and centrifuged; the supernatant fluid is used as antigen. This method was described by Krumwiede and Noble[20] for the preparation of a pneumococcus precipitinogen.

Method 16.—Bacteria are secured by washing from solid media with distilled water and 25 cc. of antiformin added to each 100 cc. This mixture is allowed to stand at room temperature for an hour or two until almost clear, when it is centrifuged, the supernatant fluid neutralized to litmus and filtered through a Berkefeld filter. The filtrate is now cooled to 8° C. in a freezing mixture and slowly added to 1 liter of cold absolute alcohol. The precipitate is collected on paper and washed twice more with cold alcohol and twice with cold ether. The final suspension is filtered through paper, the residue dried in a vacuum, sealed in small tubes and sterilized in an autoclave for one-half hour. This method has been described by Dean[21] for the preparation of typhoid antigen. When used 0.01 gm. of powder was placed in 10 cc. of sterile saline solution.

Preparation of Group IV Antigens.—*Method* 17.—Bacteria are thoroughly washed, dried and pulverized into a fine powder. One gram of this powder is triturated with 100 cc. of a 25 per cent solution of glycerin in sterile distilled water and slowly boiled for one hour in a flask with a reflux condenser. The material is now set aside for a few hours for heavy clumps to settle and the supernatant fluid stored in a refrigerator and used as antigen. This is one of the methods described by Petroff[22] for the preparation of an antigen of tubercle bacilli.

Method 18.—Cultures are washed from solid media or secured by centrifuging broth cultures and the sediment dried in Petri dishes in a desiccator over calcium chloride or in an incubator. The dried material is weighed as required and each 0.01 gm. ground in a mortar with 0.1 gm. of crystals of sodium chloride 10 cc. of water containing 0.5 per cent phenol being gradually added. This yields an antigen of which each cubic centimeter contains 0.001 gm. bacterial substance in isotonic saline solution.

Method 19.—Same as Method No. 18 except that suspensions of bacteria in saline solution are treated with an equal volume of absolute ethyl alcohol. The precipitate is secured by centrifuging, dried, ground with sodium chloride suspended in water and preserved with 0.5 per cent phenol as described above in Method No. 18 (after Gay's modification of a Besredka antigen.)

Method 20.—Washed bacteria are dried, ground and suspended in saline solution in the proportion of 0.01 gm. per cc. followed by daily freezing for one hour and thawing for three days when the emulsion is preserved with 0.5 per cent phenol and used for antigen.

Preparation of Group V Antigens.—*Method* 21.—Young or old cultures are cultivated in ordinary or special broth and autoclaved for sterilization followed by removal of the cellular elements by filtration. The filtrate is used, preserved with 0.5 per cent phenol (Koch's old tuberculin and mallein).

Method 22.—The tuberculin of Besredka and Manoukline[23] employed as antigen for complement-fixation in tuberculosis is prepared in this manner, the medium being made as follows: Dilute the white of an egg with 10 volumes of sterile water and filter through a hard paper (Chardin); repeat, using the yolk in the same manner and clarify by the gradual addition of a normal solution of sodium hydroxide. Autoclave both solutions and keep separately. *Just before using*, mix 10 volumes of sterile alkaline veal infusion with 2 volumes of the sterile egg-white solution and 1 volume of the sterile egg-yolk solution; place the mixture in sterile tubes and use without further sterilization.

Method 23.—Bacteria are grown on a solid medium and removed with saline solution or secured by centrifuging broth cultures. The sediment is suspended in sterile distilled water and heated at 56° to 60° C. for two hours for sterilization and to aid autolysis; it is then placed in an incubator for twenty-four hours or shaken for the same length of time at room tem-

perature and passed through a sterile Berkefeld filter. The filtrate is heated at 56° C. for one hour on three successive days for sterilization and preserved with 0.5 per cent phenol. This is essentially the method devised by Schwartz and McNeil for preparing gonococcus antigen.[24] For use, 1 part of antigen is diluted with 9 parts of 9 per cent solution of sodium chloride.

Method 24.—Bacteria are grown on a solid medium and washed off with saline solution or secured by centrifuging or filtering broth cultures and suspending the sediment in saline solution. To this suspension is added an equal volume of 95 per cent alcohol followed by shaking for twelve hours, incubation at 37° C. for twenty-four hours and reshaking for six hours. The emulsion is now passed through sterile paper and a Berkefeld filter, the filtrate being used as antigen. This is a method described by Craig[25] for the preparation of an antigen of tubercle bacilli, but no longer recommended by him.

Preparation of Group VI Antigens.—*Method* 25.—Young cultures of bacteria grown on solid media are removed and suspended in distilled water (about 10 cc. being used for each 20 square inches). To this suspension is added an equal volume of 95 per cent ethyl alcohol followed immediately by centrifugalization. The precipitate is again suspended in alcohol and centrifuged; the precipitate is now suspended in ether, the original volume of the suspension being reduced to one-half each time. After removal of the supernatant ether the last traces of ether adhering to the precipitate is removed by vacuum. The bacterial mass is now dried over phosphorus pentoxide *in vacuo* for three days and stored in amounts of about 0.05 gm. in "H" tubes with phosphorus pentoxide *in vacuo*. This method was described by Hitchens and Hanson[26] for the preparation of a stable antigen of meningococci.

Method 26.—Twenty-four-hour growths of bacteria on solid medium are removed and dried in sterile Petri dishes at 53° to 56° C. The scaly mass is ground in a mortar while being moistened with chloroform followed by small additions of ether. The grinding is maintained until the last addition of ether has evaporated and a fine buff-colored powder is obtained. This is now suspended in a mixture of equal parts of chloroform and ether and shaken for four to six hours. After settling, the supernatant fluid is removed and the residue shaken up three or four times with ether. After decanting or filtering away the last ether the powder is dried in an incubator at 56° C.; likewise the residue on the filter paper which is now recovered. The powder is now placed in sterile saline solution containing 0.5 per cent phenol (25 cc. for each 0.5 gm. of residue at the start), shaken for twelve to eighteen hours and used as antigen. This method was described by Small[27] for the preparations of antigens of typhoid, paratyphoid and dysentery bacilli, pneumococci and meningococci.

Method 27.—Broth cultures (tubercle bacilli grown for three weeks in glycerin broth) are sterilized by heating in an Arnold sterilizer for one hour followed ky filtering through paper. To each volume of residue is added 10 volumes of alcohol, shaken by hand and extracted in a refrigerator for two weeks. This emulsion is then filtered through paper and the filtrate discarded, the residue being washed in absolute alcohol, secured by centrifugalization and washed twice with ether. The residue is now dried in the centrifuge tube over night in an incubator and each gram emulsified in 200 cc. of saline solution, heated for one hour at 80° C. and kept in a refrigerator as stock antigen. This method was described by Wilson for the preparation of an antigen of tubercle bacilli.[28]

Method 28.—Bacteria are grown on a solid medium and removed with phenolized saline solution exercising care against including any of the

medium; or the bacteria are removed from fluid medium by filtering or centrifuging. The bacterial emulsion is centrifuged, placed in a sterile mortar and dried in an incubator at 55° C. over night. The dried material is now moistened with small additions of ether and ground to fine powder. The powder is placed in a shaking bottle with 50 parts by volume of a mixture of equal parts of ether and absolute ethyl alcohol, tightly stoppered and shaken mechanically for twenty-four hours followed by placing in an incubator for twenty-four hours. The bacterial residue is dried by fanning or in an incubator for a few hours and suspended in a sufficient amount of phenolized distilled water. This emulsion is heated at 60° C. for two hours followed by shaking mechanically for twenty-four hours.

Method 29.—Besredka's tuberculin after the extraction of the lipins by precipitating with 10 volumes of absolute alcohol, centrifuging and suspending the sediment in saline solution. This is one of the methods advocated by Bronfenbrenner and his associates[29] for the preparation of an antigen of Besredka's tuberculin.

Preparation of Group VII Antigens. —*Method* 30.—Partial antigens of Deyke and Much starting with the residue remaining after the filtration of the lactic acid-water autolysate. The residue is extracted with 100 cc. of absolute ethyl alcohol at 0° C. for twenty-four hours and filtered ("Filtrate A. E." and stated to contain various fatty acids and lipoids). The residue is again extracted with the same amount of alcohol at 37° C. for twenty-four hours and filtered ("Filtrate A. F." and stated to contain various fatty acids and lipoids). The residue is again extracted with the same amount of alcohol at 56° C. for twenty-four hours and filtered ("Filtrate A. G." and stated to contain various fatty acids and lipoids). The residue is again extracted with the same amount of alcohol at 78° C. for twenty-four hours and filtered ("Filtrate A. H." and stated to contain various fatty acids and lipoids). The residue is extracted with 100 cc. of ether at room temperature for twenty-four hours and filtered ("Filtrate A. I." and stated to be chiefly composed of neutral fats). The residue is now suspended in 100 cc. of saline solution ("tuberculo-albumen").

Method 31.—The protein-free lipoid extracts prepared by the method of Warden.[30]

A COMPARATIVE STUDY OF BACTERIAL ANTIGENS.

Not all of the above methods could be used in the preparation of antigens of tubercle, glanders and typhoid bacilli and gonococci but the majority were prepared and tested as shown in Tables 11 to 14 in an effort to arrive at some sort of reliable conclusion on what constitutes the best method or at least what are the main principles in the preparation of bacterial antigens with the highest antigenic indices.

All were titrated for hemolytic, anticomplementary and antigenic activities according to the technique described in Chapter XXXII, summarized as follows:

(*a*) **Hemolytic Titrations.**—In the hemolytic titrations each antigen was used in constant dose of 0.5 cc. undiluted and in dilutions varying from 1 to 2 to 1 to 96. Sheep corpuscles were employed and the smallest amount of antigen (0.5 cc. of the highest dilution) producing the first trace of hemolysis was recorded as the *hemolytic unit.*

(*b*) **Anticomplementary Titrations.**—In the anticomplementary titrations a similar series of dilutions of each antigen were employed; antigen and complement were incubated at 6° to 8° C. for eighteen hours followed by ten minutes in a water-bath and the addition of hemolysin and corpuscles and the secondary incubation. The smallest amount of antigen (0.5 cc. of the highest dilution) just beginning to produce inhibition of hemolysis (± or +) was recorded as the *anticomplementary unit.*

With some antigens these titrations were repeated with a primary incubation of one hour in a water-bath at 38° C.

(*c*) **Antigenic Titrations.**—In the antigenic titrations each antigen was used in constant dose of 0.5 cc. of dilutions varying from 1 to 2 to as high as 1 to 5000, depending upon the antibody strength of the respective immune sera. Antigen, complement and immune serum were incubated at 6° to 8° C. for eighteen hours followed by ten minutes in a water-bath and the addition of hemolysin and corpuscles and secondary incubation. The smallest amount of antigen (0.5 cc. of the highest dilution) just producing total inhibition of hemolysis (++++) was recorded as the *antigenic unit.*

With some antigens these titrations were repeated using a primary incubation of one hour in a water-bath at 38° C.

Various immune sera were employed. I may mention here, however, that with rabbit immune sera precautions were taken against the non-specific reactions sometimes produced by normal rabbit sera.

Practically all of the antigens were likewise titrated with refrigerator and water-bath incubations by using each antigen in constant dose of one-third its anticomplementary unit with varying amounts of immune sera. The results served as a cross check upon these observed in the regular antigenic titrations.

Antigenic Index.—*The antigenic index* is the relation of the antigenic unit to the anticomplementary unit and is obtained by the fraction: $\frac{\text{antigenic unit}}{\text{anticomplementary unit}}$ = antigenic index. For example the anticomplementary unit of a tuberculosis antigen was 0.5 cc. of 1 to 4; the antigenic unit was 0.5 cc. of 1 to 16; the antigenic index was expressed $\frac{16}{4} = 4$.

Some antigens are remarkably free of anticomplementary activity but likewise of antigenic sensitiveness; others may be strongly anticomplementary and free or nearly so of antigenic sensitiveness. The best antigens are those free or nearly so of anticomplementary activity and possessing a high degree of antigenic sensitiveness constituting "long range" antigens. Either property alone means little or nothing but the relation of the two is of prime importance and is conveniently expressed as the "antigenic index."

TABLE 11.—COMPARATIVE *Antigenic Indices* OF ANTIGENS OF TUBERCLE, TYPHOID AND GLANDERS BACILLI AND GONOCOCCI.

Antigens of.	Group I				Group II.				Group III									Group IV.		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
B. tuberculosis	5*	3	0	0	0	0	3	4	2	8	3	1.5	2	2	2	..†	7	4	2.5	2.5
B. mallei	0‡	0	0	20	40	15	20	60	10	0	15	0	30	30	20	60	80	30	8	80
B. typhosus	20	5	15	5	8	60	10	60	40	0	20	0	4	60	2	6	80	15	8	60
Gonococcus	0	0	0	0	8	5	8	15	20	0	4	0	5	5	8	0	15	8	8	10

Antigens of.	Group V.				Group VI.					Group VII.					
	21	22	23	24	25	26	27	28	29	30 A. E.	30 A. F.	30 A. G.	30 A. H.	30 A. I.	30 T. A.
B. tuberculosis	5	2	0	2	5	2	3	5	2	3	2	1.3	1.3	2	2
B. mallei	24	—	2	10	20	40	30	30	0	0	0	0	0	0	0
B. typhosus	0	0	2	4	4	60	80	10	0	0	0	0	0	0	0
Gonococcus	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0

* 5 = This means that the antigenic unit was an amount of antigen 5 times less than the anticomplementary unit.
† — = No index, *i. e.*, the anticomplementary and antigenic units were equal.
‡ 0 = Antigen not made.

Examination of Table 11 shows how the antigenic indices of different preparations of the same microörganism varied, frequently within wide limits. In this connection it may be stated that all of the different antigens of a given bacterium were prepared within a period of two weeks and *titrated at the same time with the same immune serum* in order to reduce as far as possible other variable factors and to bring out the differences ascribable to the methods of preparing the antigens.

Results.—As shown by Howell and Anderson[31] with antigens of streptococci, pneumococci and meningococci the method employed for the preparation of antigen has an important bearing upon bacterial complement-fixation and this general conclusion has been amply confirmed by our studies with tubercle, typhoid and glanders bacilli and the gonococcus.

1. As a general rule suspensions of bacteria in saline solution were superior as antigens to suspensions in fluid culture medium (Group I *vs.* Group II antigens.) The latter were somewhat more anticomplementary than the former which reduces their antigenic indices. The latter are likewise more hemolytic due to extractives in the broth medium.

2. Suspensions of whole or disintegrated bacteria were generally superior as antigens to filtrates.

3. Suspensions of dried and pulverized bacteria were superior as antigens to suspensions of whole bacteria and completely autolyzed or dissolved bacteria.

4. Removal of some of the bacterial lipoids by ether, alcohol and chloroform did not materially reduce the antigenic indices and practical value of bacterial antigens.

5. As a general rule bacterial antigens prepared of suspensions in saline solution or unfiltered suspensions of dried and thoroughly pulverized bacteria were superior to other methods.

THE DISINTEGRATION OF BACTERIA AND FUNGI UNDER VARIOUS CONDITIONS IN RELATING TO THE PREPARATION OF ANTIGENS FOR COMPLEMENT-FIXATION TESTS.

But I found upon microscopic examinations of some antigens of the class based upon complete autolysis or disintegration for the endotoxins that disintegration of the cells was only partial and for this reason did not permit an accurate comparison of the properties of antigens composed of whole bacteria in suspension with antigens of completely disintegrated bacteria. In other words the methods commonly employed for disintegration of bacterial cells in the preparation of antigens may be inadequate and result in only partial disintegration.

With the assistance of Miss Trist I have sought (1) to make a comparative study of the rate and thoroughness of different methods for the disintegration of bacteria in the preparation of bacterial antigens and (2) to compare the hemolytic, anticomplementary and antigenic properties of these antigens of disintegrated bacteria with saline suspensions of the whole microörganisms.

Methods Employed for Disintegration of Bacteria. –Five methods are commonly employed for securing the "endotoxins" of bacteria by autolysis or disintegration: (1) By suspension in water (hypotonic solutions) at low temperatures; (2) by suspension in water at high temperatures; (3) by suspension in alkaline solutions as antiformin and water; (4) by alternate freezing and thawing and (5) by drying and grinding.

Each of these five methods for producing disintegration were employed with the following microparasites: *B. diphtheria* (Park-Williams No. 8); *B. typhosus* (Hopkin's strain); *B. tuberculosis* (human); *Pneumococcus* (Type 1); *Meningococcus* (Type 1); *Trichophyton rosaceum* (ring-worm). These were selected as representatives of Gram-positive and negative bacilli and cocci, an acid fast bacillus and a pathogenic fungus.

Ten antigens were prepared of each of these according to the following methods:

1A. Suspensions in distilled water (about 1,000,000,000 per cc.) containing 0.25 per cent phenol and kept in a refrigerator at 6° to 8° C. until the Gram-positive microörganisms became Gram-negative and all had undergone complete disintegration.

1B. Same as 1A except that the suspensions were kept at room temperature.

1C. Same as 1A except that the suspensions were kept in an incubator at 38° C.

1D. Same as 1A except that the suspensions were kept in a water-bath at 60° C.

1E. Same as 1A except that the suspensions were kept in a water-bath at 80° C. (with reflux condensers.)

1F. Same as 1A except that the suspensions were kept in a bath of boiling water (with reflux condensers).

2A. Suspensions in 5 per cent antiformin in water were kept in a bath of boiling water until Gram-negative and completely dissolved.

3A. Suspensions in phenolized water were frozen in ice and salt mixtures for two hours followed by thawing in a thermostat for twenty-two hours; repeated a sufficient number of times until Gram-positive microörganisms became completely Gram-negative and had undergone complete disintegration.

4A. Suspensions in saline solution were thoroughly centrifuged and the sediment rapidly dried at 38° C. followed by grinding to fine powders with crystals of sodium chloride and gradual suspen-

sions of the powders in sufficient phenolized water to yield isotonic solutions.

For comparison with antigens of whole bacilli suspended in saline solution, the following antigen was freshly prepared of each microorganism.

5A. Suspensions in saline solution (about 1,000,000,000 per cc.), shaken for one hour, heated at 60° C. for one hour and preserved with 0.25 per cent phenol.

All of these antigens were then titrated at the same time for hemolytic, anticomplementary and antigenic activity.

Rate of Autolysis in Water at Temperatures Varying from 8° to 100° C.—In the methods commonly employed for the preparation of autolysate antigens, suspensions are made in distilled water and heated at 60° to 80° C. for a few hours on successive days. These measures, however, are inadequate.

As shown in Table 12, the diphtheria bacillus required at least nine hours to become totally Gram-negative at temperatures from 60° to 100° C. and the pneumococcus required forty to fifty hours, before all were Gram-negative, which may be interpreted as the earliest indication of autolytic changes. At temperatures from 8° to 38° C. the suspensions of diphtheria bacilli were not rendered completely Gram-negative in less than seventy-two hours and the pneumococcus required as much as three hundred to five hundred hours. Of course some microörganisms in the respective suspension became Gram-negative at earlier periods, the above periods referring to the approximate times required for *all* to become Gram-negative.

Surprisingly long periods were required for autolysis to the stage when only granular débris or complete solution were observed by microscopic examination. At temperatures varying from 8° to 38° C. the following periods were required:

Diphtheria bacillus	1000 to 1400 hours
Pneumococcus	1000 to 1200 hours
Typhoid bacillus	600 to 700 hours
Tubercle bacillus	1800 to 2000 hours
Meningococcus	1000 to 1200 hours
Trichophyton rosaceum	600 to 800 hours (for mycelia; spindles only partially disintegrated)

At temperatures varying from 60° to 100° C. the following periods were required:

Diphtheria bacillus	57 to 106 hours
Pneumococcus	48 to 177 hours
Typhoid bacillus	45 to 60 hours
Tubercle bacillus	80 to 240 hours
Meningococcus	118 to 209 hours
Trichophyton rosaceum	90 to 130 hours (for mycelia; spindles only partially disintegrated)

TABLE 12.—TIME REQUIRED FOR THE COMPLETE DISINTEGRATION OF VARIOUS BACTERIA AND FUNGI.

Methods.	Gram + to gram −		Time required for complete disintegration of:					
	B. diphtheriæ.	*Pneumococcus.*	*B. diphtheriæ.*	*B. typhosus.*	*B. tuberculosis.*	*Pneumococcus.*	*Meningococcus.*	*Trichophyton.**
1A Water at 6 to 8° C.	72 hrs.	500 hrs.	1400 hrs.	700 hrs.	2000 hrs.	1200 hrs.	1200 hrs.	800 hrs.
1B Water at 20 to 22° C.	72 hrs.	400 hrs.	1200 hrs.	700 hrs.	2000 hrs.	1000 hrs.	1200 hrs.	800 hrs.
1C Water at 38° C.	60 hrs.	300 hrs.	1000 hrs.	600 hrs.	1800 hrs.	1000 hrs.	1000 hrs.	600 hrs.
1D Water at 60° C.	11 hrs.	54 hrs.	106 hrs.	60 hrs.	240 hrs.	177 hrs.	209 hrs.	130 hrs.
1E Water at 80° C.	9 hrs.	45 hrs.	71 hrs.	60 hrs.	200 hrs.	135 hrs.	130 hrs.	120 hrs.
1F Water at 100° C.	9 hrs.	40 hrs.	57 hrs.	45 hrs.	80 hrs.	48 hrs.	118 hrs.	90 hrs.
2A 5% antiformin at 100° C.	At once	At once	1 hr.	5 hrs.	7 hrs.	1 hr.	At once	18 hrs.
3A Freezing and thawing	1 time	6 times	16 times	8 times	26 times	8 times	20 times	17 times
4A Drying and grinding	complete	complete	almost complete	almost complete	almost complete	almost complete	almost complete	complete

* Refers to disintegration of mycelia only; the spindles were only partially disintegrated.

As a general rule autolysis or disintegration were enhanced by the higher temperatures. The differences in rapidity at refrigerator (8° C.), room (20° to 22° C.) and incubator (38° C.) temperatures, however, were not marked, but at temperatures from 60° C. to boiling (100° C.) disintegration was more rapid.

The rate of disintegration varies, therefore, with different bacteria and even among bacteria of the same species; for this reason fixed times cannot be stated but in the preparation of autolysate antigens direct microscopic examination should be made and the autolysis continued until all microörganisms are reduced to a state of granular débris or complete solution.

Rate of Autolysis in Alkaline Solutions.—The addition of 5 per cent antiformin to water greatly increases the rate of disintegration. Gram-positive bacteria as the diphtheria bacillus and pneumococcus become Gram-negative in a few minutes at room temperature; when suspensions of the following bacteria in water-antiformin were boiled complete disintegration occurred as follows:

Diphtheria bacillus	1 hour
Pneumococcus	1 hour
Typhoid bacillus	5 hours
Tubercle bacillus	7 hours
Meningococcus	Few minutes
Trichophyton rosaceum	18 hours

Rate of Autolysis by Freezing and Thawing.—When suspensions of diphtheria bacilli and pneumococci in water are frozen for two hours and then allowed to thaw and remain at 38° C. for twenty-two hours, the former became Gram-negative while the latter required a repetition of this process eight times. For complete disintegration of all bacteria the following "times" were required for the different microparasites:

Diphtheria bacillus	16 times
Pneumococcus	8 times
Typhoid bacillus	26 times
Meningococcus	20 times
Trichophyton rosaceum	17 times

Rate of Disintegration by Drying and Grinding.—When the diphtheria bacillus and pneumococcus were dried at 38° C. and thoroughly ground into a fine powder, all of the fragments were found to be Gram-negative. This process resulted in marked disintegration of all bacteria and the fungus included in this study, the tubercle bacillus proving the most resistant and difficult of disintegration.

When these powders were extracted with ether and chloroform, disintegration became complete to the stage of granular débris with all the microparasites studied excepting the tubercle bacillus.

TABLE 13.—ANTICOMPLEMENTARY AND ANTIGENIC UNITS.

Methods employed for antigens.	*B. diphtheriæ.*		*B. typhosus.*		*B. tuberculosis.*		*Pneumococcus.*		*Meningococcus.*		*Trichophyton.*	
	Anticomplementary.	Antigenic.*	Anticomplementary.	Antigenic.†	Anticomplementary.	Antigenic.‡	Anticomplementary.	Antigenic.§	Anticomplementary.	Antigenic.**	Anticomplementary.	Antigenic.††
1A Water at 6 to 8° C.	1:6	1:18	1:16	1:32	1:8	1:32	1:2	1:4	1:4	1:16	1:4	1:8
1B Water at 20 to 22° C.	1:4	1:16	1:16	1:32	1:8	1:32	1:2	1:8	1:4	1:16	1:4	1:8
1C Water at 38° C.	1:4	1:16	1:16	1:32	1:8	1:32	1:2	1:2	1:4	1:16	1:4	1:8
1D Water at 60° C.	1:4	1:8	1:16	1:32	1:8	1:32	1:2	1:2	1:4	1:16	1:2	1:4
1E Water at 80° C.	1:2	1:8	1:16	1:32	1:8	1:32	1:2	1:2	1:4	1:24	1:2	1:4
1F Water at 100° C.	1:4	1:16	1:40	1:12	1:8	1:32	1:2	1:2	1:2	1:8	1:2	1:6
3A Freezing and thawing	1:2	1:12	1:40	1:32	1:8	1:16	1:2	1:16	1:16	1:32	1:2	1:4
4A Drying and grinding	1:2	1:16	1:16	1:256	1:2	1:24	1:2	1:8	1:4	1:32	1:2	1:10
5A Suspension in saline (control)	1:2	1:10	1:20	1:120	1:8	1:48	1:2	1:8	1:4	1:16	1:2	1:12

* Determined with 0.05 cc. horse antitoxic serum.
† Determined with 0.0025 cc. rabbit antityphoid serum.
‡ Determined with 0.025 cc. calf antituberculosis serum.
§ Determined with 0.1 cc. horse antipneumococcus serum (Type 1).
** Determined with 0.05 cc. horse antimeningococcus serum.
†† Determined with 0.0025 cc. rabbit antiserum.

The amount of time required for complete disintegration varies considerably not only among different bacteria but to some extent among bacteria of the same species; the periods given in this paper for the different methods are approximately correct and show that some of the methods commonly employed for preparing antigens of disintegrated bacteria are inadequate for this purpose. Alkaline solutions (2 to 5 per cent sodium hydroxide) and drying followed by thorough grinding bring about complete disintegration more quickly than other methods. *In the preparation of all autolysate antigens the degree of disintegration should be controlled by direct microscopic examinations; in the antiformin preparations "clearing" of the suspensions may be sufficient.*

Comparative Properties of Autolysate Antigens versus Suspensions of Whole Bacteria.—The results of anticomplementary and antigenic titrations are given in Table 13. All antigens were free of hemolytic activity, those prepared with antiformin (2a) having been omitted from this part of the study. The antigenic indices which show at a glance the comparison of antigenic activity, are shown in Table 14; 4 in this table means that the antigenic unit was one-fourth the anticomplementary unit.

Antigens 5A were the suspensions of the respective microörganisms in saline solution, for comparison with the antigens 1A to 4A composed of disintegrated bacteria.

TABLE 14.—ANTIGENIC INDICES.

Methods employed for antigens.	*B. diphtheriæ.*	*B. typhosus.*	*B. tubercle.*	*Pneumococcus.*	*Meningococcus.*	*Trichophyton rosaceum*
1A Water at 6 to 8° C. . . .	3	2	4	2	4	2
1B Water at 20 to 22° C. . .	4	2	4	4	4	2
1C Water at 38° C.	4	2	4	..	4	2
1D Water at 60° C.	2	2	4	..	4	2
1E Water at 80° C.	4	2	4	..	6	2
1F Water at 100° C.	4	3	4	..	4	3
3A Freezing and thawing . .	6	8	2	8	2	2
4A Drying and grinding . . .	8	16	12	4	8	5
5A Suspension in saline (control)	5	6	6	4	4	6

The results may be summarized as follows:

1. None of the autolysates in water proved superior antigens, but antigens of fairly good properties resulted with *B. diphtheriæ*, *B. tuberculosis* and the meningococcus.

2. Antigens of disintegrated bacteria prepared by drying and grinding proved superior to others with diphtheria, typhoid and tubercule bacilli and the meningococcus.

3. Antigens prepared by freezing and thawing proved best for the pneumococcus and next best for *B. diphtheriæ* and *B. typhosus*.

4. Antigens prepared of suspensions of bacteria in saline solution proved best for *Trichophyton rosaceum* and generally satisfactory with the bacteria particularly the tubercle bacillus, typhoid bacillus and diphtheria bacillus. *Therefore for the preparation of antigens of bacteria and possibly also of the pathogenic fungi and molds for complement-fixation tests, suspension of dried and ground organisms in saline solution or suspensions of whole fresh organisms in saline solution are generally most satisfactory and yield the highest antigenic indices.*

Not only were the antigenic indices of these antigens generally higher than antigens of completely dissolved bacteria, but the complement-fixation reactions appeared to be sharper and more easily read. It would appear, therefore, that the presence of the whole bacterial cell or its fragments enhances the value of antigens of bacteria and fungi but further investigations with assistance of Miss Yagle and Miss Trist were undertaken to study this proposition.

A COMPARATIVE STUDY OF THE PROPERTIES OF BACTERIAL SUSPENSIONS VERSUS FILTRATES, CENTRIFUGATES AND DISTILLATES AS ANTIGENS FOR COMPLEMENT-FIXATION TESTS.

For this purpose three kinds of bacterial antigen were prepared: suspensions of whole bacteria in saline; suspension and solutions of dried, ground and disintegrated bacteria and suspensions and solutions of bacteria autolyzed in water. Portions of each of these three antigens of the respective bacteria were thoroughly centrifuged, passed through earthen filters and distilled. These centrifugates, filtrates and distillates were then titrated at the same time as the whole suspensions and solutions and their properties compared.

Antigens were prepared of gonococci, diphtheria, glanders and tubercle bacilli, pneumococci, and meningococci and *Trichophyton rosaceum* by cultivation on appropriate solid media followed by removal with saline solution, shaking the suspensions for two hours and heating at 60° to 80° C. for one hour.

Portions of these suspensions were centrifuged at high speed for several hours and the supernatant fluids employed.

Other portions were passed through neutral, dry and sterile Kitasato earthen filters and the filtrates employed.

A second set of antigens were prepared by drying the bacteria and grinding thoroughly. These powders were suspended in saline solution and portions centrifuged and filtered as above. These yielded antigens of disintegrated bacteria in suspension as well as filtrates and centrifugates of these.

A third set of antigens were prepared by completely autolyzing or disintegrating the bacteria in water at 80° C. Portions were centri-

fuged and filtered. These preparations yielded antigens of completely dissolved bacteria as well as filtrates and centrifugates of these.

Some of these antigens including a cholesterolized alcoholic extract of heart and an extract of acetone-insoluble lipoids of heart, were distilled once and the distillates employed as antigens.

All of these antigens were titrated for hemolytic, anticomplementary and antigenic activities at the same time and by the same technique. None were hemolytic in 0.5 cc. of undiluted antigen (the largest amount of each tested) and these results are omitted from the tables.

Summary of Results.—Table 15 gives the anticomplementary and antigenic units and antigenic indices of the suspension antigens and the filtrates, centrifugates and distillates of these. Table 16 gives the results with the antigens of disintegrated and autolyzed bacteria and the filtrates and centrifugates of these.

1. Whole bacterial antigens, that is, suspensions of fresh bacteria in saline solution sterilized by heat, have generally yielded higher antigenic indices than filtrates and centrifugates of these (Table 15). Centrifugates ranked next followed by the filtrates. The distillates were entirely lacking in specific antigenic activity.

A general average of the antigenic indices of the eleven bacterial antigens shown in Table 15 permits expressing these comparative values numerically as follows:

Antigenic index of suspensions	= 5
Antigenic index of centrifugates of these	= 4
Antigenic index of filtrates of these	= 3
Antigenic index of distillates of these	= −

2. Dried and ground bacteria in saline solution yielded higher antigenic indices than the centrifugates and filtrates of these (Table 16). Centrifugates ranked second followed by filtrates. Distillates were not prepared.

The general averages may be expressed numerically as follows:

Antigenic index of suspension	= 7
Antigenic index of centrifugates of these	= 5
Antigenic index of filtrates of these	= 2.5

3. Autolysates of bacteria in water yielded higher antigenic indices than the centrifugates and filtrates of these (Table 16). Centrifugates ranked second followed by filtrates. Distillates were not prepared.

The general averages may be expressed numerically as follows:

Antigenic index of autolysates	= 3
Antigenic index of centrifugates of these	= 2.6
Antigenic index of filtrates of these	= 1.6

TABLE 15.—THE INFLUENCE OF FILTRATION, CENTRIFUGALIZATION AND DISTILLATION UPON THE PROPERTIES OF ANTIGENS OF SUSPENSIONS OF MICROÖRGANISMS IN SALINE SOLUTION, SHAKEN FOR TWO HOURS AND HEATED FOR ONE HOUR AT 60° C. OR 80° C. ALSO THE EFFECT OF DISTILLATION UPON ALCOHOLIC EXTRACTS OF HEART MUSCLE.

Microörganism.	Suspensions.			Filtrates.			Centrifugates.			Distillates.		
	Anticomplementary.	Antigenic.	Index.	Anticomplementary.	Antigenic.	Index.	Anticomplementary.	Antigenic.	Index.	Anticomplementary.	Antigenic.	Index.
Gonococcus No. 1	1:6	1:60	10	1:2	1:4	2	1:6	1:2	3	1:2	1:2	—
Gonococcus No. 2	1:10	1:25	2.5	1:6	1:8	1.3	1:8	1:16	2	0	0	0
Gonococcus No. 3	1:16	1:32	2	1:4	1:20	5	1:4	1:16	4	0	0	0
Gonococcus No. 4	1:8	1:64	8	1:2	1:6	3	1:8	1:40	5	0	0	0
B. diphtheriæ	1:2	1:4	2	undil.	1:2	2	1:4	1:6	1.5	0	0	0
B. tuberculosis No. 1	1:8	1:48	6	1:4	1:16	4	1:2	1:16	8	1:2	1:2	—
B. tuberculosis No. 2	1:16	1:48	3	1:2	1:4	2	1:2	1:6	3	1:4	1:4	—
Pneumococcus	1:2	1:8	4	undil.	1:3	3	1:2	1:6	3	0	0	0
Meningococcus	1:4	1:16	4	1:2	1:16	8	1:3	1:12	4	0	0	0
Trichophyton	1:2	1:8	4	undil.	1:2	2	1:2	1:4	2	0	0	0
B. typhosus No. 1	1:4	1:16	4	1:2	1:6	3	1:4	1:16	4	1:2	1:2	—
B. typhosus No. 2	1:4	1:64	16	undil.	1:4	4	1:2	1:32	16	0	0	0
Cholest. alc. heart	1:5	1:400	80	0	0	0	0	0	0	1:4	1:4	—
Acetone-insoluble lipoids	1:5	1:300	60	0	0	0	0	0	0	1:4	1:4	—

TABLE 16.—THE INFLUENCE OF FILTRATION AND CENTRIFUGALIZATION UPON THE PROPERTIES OF ANTIGENS OF *Completely Disintegrated Bacteria.*

Microörganism.	Process of disintegration.	Whole antigens.			Filtrate antigens.			Centrifugate antigens.		
		Anticomplementary.	Antigenic.	Index.	Anticomplementary.	Antigenic.	Index.	Anticomplementary.	Antigenic.	Index.
Gonococcus	Dried and ground	1:16	1:64	4	1:2	1:6	3	1:16	1:32	2
Gonococcus	Water at 80° C.	1:10	1:30	3	1:2	1:4	2	1:6	1:12	3
B. diphtheriæ	Dried and ground	1:2	1:4	2	undil.	undil.	—	undil.	1:2	2
B. diphtheriæ	Water at 80° C.	1:8	1:16	2	1:2	1:2	—	1:8	1:12	1.5
B. typhosus	Dried and ground	1:16	1:64	4	1:2	1:10	5	1:12	1:64	5
B. typhosus	Water at 80° C.	1:10	1:30	3	1:8	1:16	2	1:8	1:12	1.5
B. tuberculosis	Dried and ground	1:4	1:64	16	1:3	1:6	2	1:2	1:32	16
B. tuberculosis	Water at 80° C.	1:16	1:32	2	1:2	1:3	1.5	1:12	1:30	2.5
B. mallei	Dried and ground	1:4	1:64	16	1:2	1:3	1.5	1:4	1:64	16
B. mallei	Water at 80° C.	1:4	1:32	8	1:2	1:3	1.5	1:4	1:32	8
Pneumococcus	Dried and ground	1:2	1:12	6	1:2	1:3	1.5	1:2	1:4	2
Pneumococcus	Water at 80° C.	1:3	1:6	2	1:2	1:3	1.5	1:3	1:6	2
Meningococcus	Dried and ground	1:4	0	0	1:2	0	0	1:2	0	0
Meningococcus	Water at 80° C.	1:4	0	0	1:2	0	0	1:4	0	0

TABLE 17.—SUMMARY SHOWING *Average* ANTICOMPLEMENTARY AND ANTIGENIC UNITS AND ANTIGENIC INDICES OF TWELVE BACTERIAL ANTIGENS AND THE CENTRIFUGATES AND FILTRATES OF THESE.

Kind of antigen.	Whole antigens.			Centrifugates.			Filtrates.			Distillates.		
	Anticomplementary units.	Antigenic. units.	Indices.	Anticomplementary units.	Antigenic units.	Indices.	Anticomplementary units.	Antigenic units.	Indices.	Anticomplementary units.	Antigenic units.	Indices
Suspensions of bacteria in saline solution heated at 60 to 80° C.	1:7	1:33	5	1:4	1:15	4	1:2	1:7	3	1:2.5	1:2.5	—
Dried and ground bacteria in saline solution	1:7	1:46	7	1:6	1:33	5	1:2	1:5	2.5	0	0	0
Autolyzed bacteria in water	1:8.5	1:24	3	1:7	1:18	2.6	1:3	1:5	1.6	0	0	0

4. The differences between whole antigens and centrifugates and filtrates of these were more decided with suspensions of whole bacteria than with the antigens of disintegrated and autolyzed bacteria (Table 17).

5. When bacterial antigens are thoroughly centrifuged the supernatant fluids (centrifugates) are generally less anticomplementary than whole antigens; there is, however, slightly greater reduction in proportion of antigenic activity which slightly reduces the antigenic indices of the centrifugates as compared with whole antigens (Table 17). The centrifugates obtained by washing bacteria from solid media into saline solution and centrifuging at once at high speed constitute the so-called "ecto-antigens" because they are believed to be neither endocellular nor extracellular in origin, but derived from the ectoplasm of the cells. With the typhoid and glanders bacilli they sometimes yield good antigens; for example, the *antigenic indices* obtained with a typhoid preparation were as follows:

Ecto-antigen	4
Suspension in phenolized saline (unheated)	4
Broth culture (phenolized and unheated)	2

Similar antigens prepared of human and bovine tubercle bacilli removed from glycerin-agar slant cultures, however, were almost devoid of antigenic activity.

6. When bacterial antigens are filtered in such manner as to remove whole bacteria and probably larger fragments, as when Berkefeld filters of average density are employed, the filtrates have a decidedly less anticomplementary activity as compared with unfiltered antigen; there is, however, a decided decrease in antigenic activity so that the antigenic indices of filtrate antigens are slightly less then the indices of centrifugates and decidedly less than the indices of whole antigen (Table 17).

7. When bacterial antigens are distilled once, the distillates have a slightly reduced anticomplementary activity as compared with whole antigen; there is however, a complete or almost complete absence of specific antigenic activity so that distillates have practically no antigenic indices. The distillation of alcoholic tissue extracts employed in the Wassermann syphilis test removes some of the anticomplementary effects and all of the antigenic, so that distillates of these antigens are likewise devoid of antigenic indices.

It was found therefore, that in general terms the best bacterial antigens are in the order named: (1) *dried and ground organisms in saline solution;* (2) *suspensions of whole bacteria in saline solution heated at 60° to 80° C. for two hours and* (3) *centrifugates of these.*

ANTIGENIC ACTIVITY OF BACTERIAL LIPOIDS.

As previously stated some investigators regard the lipoids of bacteria as specifically and actively antigenic in the complement-fixation reaction and particularly in the case of tubercle bacillus antigens. Others regard the proteins as the specific and active antigens and others still combination products of the two. Much[32] extracted a fat called nastin from a streptothrix isolated from lepers which he thought was antigenic both *in vivo* and *in vitro*. Thiele and Embleton[33] found that the phosphatides of the tubercle bacillus, which gave none of the color reactions for protein produced complement-fixing antibody in the lower animals, reacted vigorously with specific phosphatide antigen and quite definitely with the whole bacilli and with bacilli extracted with trichlorethylene to remove wax. Essentially similar results have been reported quite recently by Wadsworth and his associates with the acetone-insoluble lipoids of tubercle bacilli and Furth and Aronson[34] have also observed that tubercle bacilli of mammalian, avian and other origin contain specific alcohol-soluble substances. Inasmuch however, as their extracts were prepared with 95 per cent alcohol it is possible that enough protein was extracted to give the antigens specific characters. Lewis[35] however, states that prolonged extraction of *dried* tubercle bacilli with boiling *absolute* ethyl alcohol followed by one or more reprecipitations by chilling the hot alcoholic solution, yields a preparation very active as antigen in the complement-fixation reaction and still further emphasizing the antigenic importance of the alcohol-soluble lipoids of this organism.

In my experience *cold* alcoholic, ether and acetone extracts of tubercle, typhoid and glanders bacilli, gonococci and cultures of *Spirochæta pallida*, have proven much less antigenic than saline extracts or suspensions; furthermore, they have frequently yielded cross positive complement-fixation reactions with the sera of syphilitic individuals free of specific antibody for the antigens, which is an important objection to them. I believe it may be stated therefore that alcoholic extracts of bacteria are usually inferior to saline or watery extracts although this does not by any means indicate that the bacterial lipoids are antigenically inactive since they may be contained in larger amounts and dispersed in a more actively antigenic state in the latter than in the former extracts.

Extracts of tubercle, typhoid and cholera bacilli prepared by Warden's method, which are stated to contain the various fatty acids, have not proven antigenic in my experience. As shown in Table 18 as an example of a series of experiments, they have been much more hemolytic than control antigens of bacillary suspensions in saline solutions. Furthermore, they were almost devoid of antigenic activities in dilutions that were not hemolytic and were much

more likely to yield cross complement-fixation reactions with syphilitic sera. Aside from the complicated technique of preparation they have failed in my experience as practical antigens and indicate that the fatty acids of bacteria do not represent the maximum of antigenic activity of the bacterial lipoids.

TABLE 18.—THE COMPLEMENT-FIXING PROPERTIES OF THE FATTY ACIDS OF BACTERIA. (WARDEN METHOD.)

Organisms.	Bacillary suspensions.				Fatty acids.			
	Hemolytic unit, 0.5 cc.	Anticomplementary unit, 0.5 cc.	Antigenic unit, 0.5 cc.	Reaction with syphilitic serum.	Hemolytic unit, 0.5 cc.	Anticomplementary unit, 0.5 cc.	Antigenic unit, 0.5 cc.	Reaction with syphilitic serum.
B. tuberculosis . .	1:4	1:8	1:90	+++	1:20	*	*	*
B. typhosus . . .	1:4	1:5	1:50	− to +	1:20	*	*	++
B. cholera . . .	1:4	1:5	1:128	− to +	1:5	1:10	*	++

* Not anticomplementary or antigenic in dose of 0.5 cc. of 1 to 25 or higher; lower dilutions hemolytic.

The acetone-insoluble lipoids of tubercle, typhoid and glanders bacilli have proven more antigenic. These extracts were prepared of washed and dried bacteria by extracting with boiling ether and then with boiling alcohol; the latter were evaporated and the residues extracted with ether which was added to the primary ether and the acetone-insoluble lipoids thrown out of solution by adding 5 volumes of acetone. The residues were now redissolved in ether and acetone-free methyl alcohol, the whole technique being almost identical with my method for extracting the acetone-insoluble lipoids from powdered beef heart muscle.

As shown in Table 19 these extracts were non-hemolytic, of very low anticomplementary activity and likewise low in antigenic activity as compared with control antigens prepared by suspending 0.1 gm. of the dried bacillary residues after alcohol extractions in 20 cc. of distilled water, boiling with reflux condensers for two hours and adding sodium chloride to isotonicity.

The yield of antigen in the case of typhoid and glanders bacilli was very small and all of the extracts yielded cross complement-fixation tests with syphilitic sera.

The results have shown however, that the acetone-insoluble lipoids of the bacteria studied possessed some antigenic activity although all of my extracts contained very minute traces of protein

and thereby involved the possibility of the antigenic activity being due in part at least to the presence of protein or lipoid-protein complexes. From the practical standpoint these extracts were not superior to those prepared by much simpler and less expensive methods and their tendency to yield a higher percentage of cross complement-fixation reactions with the sera of syphilitic individuals constitute a weighty drawback to their use.

TABLE 19.—THE COMPLEMENT-FIXING PROPERTIES OF THE ACETONE-INSOLUBLE LIPOIDS OF BACTERIA.

Organisms.	Bacillary residues.				Acetone-insoluble lipoids			
	Hemolytic unit, 0.5 cc.	Anticomplementary unit, 0.5 cc.	Antigenic unit, 0.5 cc.	Reactions with syphilitic serum.	Hemolytic unit, 0.5 cc.	Anticomplementary unit, 0.5 cc.	Antigenic unit, 0.5 cc.	Reactions with syphilitic serum.
B. tuberculosis . .	Not in 1:4	Not in 1:4	1:15	+	Not in 1:4	1:10	1:20	+++
B. typhosus . .	Not in 1:4	1:8	1:600	−	Not in 1:4	1:4	1:8	±
B. mallei . . .	Not in 1:4	1:4	1:100	+	Not in 1:4	1:4	1:10	±

THE POSSIBILITY OF BACTERIAL AND OTHER ANTIGENS YIELDING FALSELY POSITIVE COMPLEMENT-FIXATION REACTIONS WITH SYPHILIS ANTIBODY.

It is now generally agreed that the complement-fixation reaction in syphilis with the antigens in common use is a non-specific fixation of complement by antibody and lipoidal substances in the extracts employed as antigens. Since bacteria and culture media employed for the cultivation of bacteria contain lipoidal substances, the question arises whether or not such antigens as those of gonococci, typhoid and tubercle bacilli, echinococcus cyst fluid and so forth contain sufficient lipoids to fix complement with syphilitic sera and thereby yield non-specific positive reactions which may be interpreted as specific bacterial complement-fixation reactions. In as much as syphilis is a widely prevalent disease and is frequently encountered during the latent stages when the serum yields positive Wassermann reactions but the individual shows none or but doubtful clinical evidences, this is a question of considerable practical importance.

In 1913, Williams, Laubaugh and myself found that saline and alcoholic extracts of *Spirochæta pallida* cultures and ascites-agar

culture medium as well as similar extracts of cultures of typhoid and cholera bacilli may yield positive complement-fixation reactions with some strongly Wassermann positive sera and that analyses of these extracts disclosed the presence of lipoids. We concluded that these complement-fixation reactions were partly due to these bacterial lipoids reacting with the syphilitic antibody and by exactly the same mechanism as complement-fixation in syphilis with the lipoids in aqueous or alcoholic extracts of heart muscle, liver or other tissues.

To the best of my knowledge no one has reported gonococcus antigens yielding positive complement-fixation reactions with the sera of syphilitics free of gonococcus infection, but there are numerous reports in literature to show that most antigens of tubercle bacilli may react with the sera of syphilitics free of tuberculosis. Bronfenbrenner found this true of Besredka's tuberculin and for this reason advised the removal of the lipins by fat solvents or the precipitation and recovery of the proteins. Miller[38] however, reported that with his antigen of suspensions of tubercle bacilli in saline solution there is almost complete absence of complement-fixation with syphilitic sera. Craig[39] has likewise reported the absence of these reactions with an alcoholic extract of egg-broth cultures of tubercle bacilli. Woods, Bushnell and Maddux[40] observed positive reactions with the sera of syphilitics using the "partial antigens" of Deyke and Much; likewise Lucke[41] with alcoholic solutions of tubercle wax, Fleisher and Ives[42] with their antigen and Corper and Sweeney[43] with autolysate antigen and Miller's bacillary preparation. von Wedel[44] has reported some positive reactions with the sera of syphilitics apparently free of tuberculosis as likewise Wadsworth and his associates with the acetone-insoluble lipoids of tubercle bacilli.

In view of the fact that the syphilis antibody may be unexpectedly present in the sera of individuals submitted for the tuberculosis, typhoid, gonococcus, echinococcus or other complement-fixation test, Miss Yagle and I have studied a large number of antigens for the purpose of ascertaining whether or not the syphilis antibody may fix complement in a non-specific manner with these antigens and if so, what technical modifications may be employed to avoid them and render a bacterial complement-fixation test specific with the serum of a syphilitic individual. This is particularly important for the serological diagnosis of active tuberculosis among syphilitics.

The technique of complement-fixation was that described in Chapter XXXII and each antigen was employed in an amount equivalent to one-third its anticomplementary unit. The following were employed:

(*a*) Gonococcus antigens prepared by methods: 5, 6, 7, 8, 10, 12, 13, 14, 15, 16, 17, 19, 20, 21, 22, 23, 24, 25, 26, and 27.

(*b*) Typhoid antigens prepared by methods: 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 13, 14, 15, 16, 17, 19, 20, 21, 22, 23, 24, 25, 26 and 27.

(*c*) Glanders antigens prepared by methods: 2, 3, 4, 5, 6, 7, 8, 10, 12, 13, 14, 15, 16, 17, 19, 20, 21, 22, 23, 24, 25, 26 and 27.

(*d*) Tuberculosis antigens prepared by methods: 2, 5, 6, 7, 8, 9A, 9B, 12, 13, 14, 15, 16, 17, 18A.E., 18A. F., 18 A. G., 18 A. H., 18 A. I., 18 T. A., 19, 20, 21, 22, 23, 24, 25, 26 and 27.

(*e*) The fluid of an echinococcus cyst of the liver.

In addition to these antigens, extracts of the bacterial fats were prepared by extracting dried and pulverized bacterial residues of each of the four species mentioned above with ether and ether-alcohol. These were evaporated, the residues dissolved in a minimal amount of ether and added to absolute ethyl alcohol. Dilutions were prepared with saline solution. Purified fats were also prepared according to the method of Warden from tubercle, typhoid and cholera bacilli.

Sera were collected from syphilitic persons with considerable care; each serum yielded positive Wassermann reactions of varying degrees. The sera employed with the tuberculosis antigens were selected from individuals free of all clinical evidences of tuberculosis and yielding negative von Pirquet skin tests. The sera employed with the gonococcus antigens were selected from males with no historical or clinical evidences of gonococcus infection. The sera employed with the typhoid antigens were from syphilitics who (according to their own statements) had never had typhoid fever or been vaccinated. No particular precautions were observed in the selection of those sera employed with the glanders and echinococcus antigens. All sera were heated at 55° C. for fifteen minutes and used in dose of 0.1 cc. with each antigen.

Summary of Results.—(*a*) *With the Gonococcus Antigens.*—The results were uniformly negative. With heavy suspensions of unwashed organisms in saline solution, slight inhibition of hemolysis was noted with some sera containing very large amounts of syphilis reagin, as from individuals in the secondary stage of syphilis. The alcohol extract of dried gonococci however, yielded some positive reactions with the syphilitic sera indicating the presence of sufficient lipoids in this extract to yield the lipotropic reaction.

(*b*) *With the Typhoid and Glanders Antigens.*—Antigens belonging to Group I (prepared according to Methods 1, 2 and 3) which were cultures in fluid culture media, occasionally yielded weakly positive reactions; these results were ascribed to the presence of lipoids from the medium in addition to the bacterial lipoids. The remaining antigens yielded uniformly negative results although with occasional sera yielding very strongly positive Wassermann reactions, slight degrees of inhibition of hemolysis were noted. The ether-alcohol extracts of these cultures yielded weakly positive reactions with the

majority of sera and the purified fats prepared by the method of Warden yielded strongly positive Wassermann reactions as did likewise the acetone-insoluble lipoids of these organisms.

(*c*) *With the Tuberculosis Antigens.*—These results were particularly interesting and have shown that many different antigens of tubercle bacilli react positively with syphilitic sera and especially with sera containing large amounts of syphilitic antibody.

The Group I antigens, which were broth cultures (three weeks or, longer) of tubercle bacilli, sterilized with heat or phenol, yielded positive reactions of varying degrees with as high as 80 per cent of sera from non-tuberculous syphilitic individuals. These results were ascribed to the relatively high content of lipoids in these antigens from both medium and bacilli and especially with egg-broth and glycerin-broth cultures.

With the Group II antigens (suspensions of tubercle bacilli in saline solution killed by heat), the results varied to some extent with the age of the cultures and culture medium. For example, unwashed bacilli obtained from filter papers after filtering eight weeks 5 per cent glycerin veal broth cultures and suspending in saline solution, yielded about 23 per cent positive reactions, whereas with suspensions of bacilli in saline obtained from ten to fourteen day cultures on slants of solid egg medium as employed in the preparation of the Miller-Zinsser and Fleisher-Ives antigens, about 12 per cent of syphilitic sera yielded positive reactions.

The Besredka tuberculin which is a filtrate of egg-broth culture, yielded about 30 per cent positive reactions.

The Group IV antigens (suspensions of bacilli autolyzed in water or disintegrated by drying and grinding) yielded about 20 per cent positive reactions.

The Group V antigens (filtrates of autolyzed bacilli and including the "partial antigens" of Deyke and Much varied considerably with the different antigens. Antigen No. 23 prepared with distilled water after the Schwartz-McNeil method of preparing gonococcus antigen, yielded about 20 per cent positive reactions. The filtrate of an alcoholic extract of the bacilli suspended in saline solution (No. 24, after Craig's method) yielded about 4 per cent positive reactions. The filtrate of the lactic acid water extract ("Filtrate A" of Deyke and Much) yielded about 30 per cent positive reactions; the first alcoholic filtrate ("A. E.") yielded about 6 per cent positive reactions but with the remaining four alcohol and ether filtrates (A.F.; A.G.; A.H. and A.I.) and the bacterial residue (tuberculo-albumen), the reactions were uniformly negative.

The Group VI antigens were of particular interest inasmuch as in their preparation lipoid solvents as ether, alcohol and chloroform were employed and these extractives discarded, the antigens (Nos. 25, 26, 27, 28 and 29) being prepared of the bacterial residues.

Antigen No. 26 (prepared after Small's method) and antigen No. 27 (prepared after Wilson's method) were of most interest. With both antigens positive reactions were observed with about 4 per cent of sera and all of these sera yielded strongly positive Wassermann reactions. With sera yielding weakly positive Wassermann reactions the results were uniformly negative with these antigens and antigen No. 28.

With the Group VII antigens including the ether-alcohol extracts of dried tubercle bacilli and the purified fats prepared by Warden's method, positive reactions were observed with almost 90 per cent of the syphilitic sera employed in this investigation; similar results were observed with the acetone-insoluble lipoids.

(*d*) *With the Echinococcus Antigen.*—As previously stated, this was the fluid removed from an echinococcus cyst of the liver; it yielded about 80 per cent positive reactions with the sera of syphilitic individuals. These reactions were undoubtedly due to the large amount of lipoids in this cyst fluid.

Therefore, the syphilis complement-fixing antibody occurring in the sera of syphilitic individuals may yield non-specific positive complement-fixation reactions with the lipoids of bacteria and culture media.

Of the antigens of typhoid and glanders bacilli, those prepared by cultures in fluid culture media are most likely to yield these reactions. Saline suspensions of these bacilli and gonococci yielded only occasional weakly positive reactions with sera containing exceptionally large amounts of syphilis antibody.

Antigens of tubercle bacilli and echinococcus fluid are especially likely to yield non-specific reactions with syphilis antibody; for this reason special precautions should be taken for the removal of the lipoids from these antigens. Fortunately the removal of the lipoids from bacterial antigens does not reduce their specific antigenic sensitiveness or practical value for complement-fixation tests and practically this precaution is required only in the case of antigens of the tubercle and other acid-fast bacteria containing exceptionally large amounts of lipoids.

THE EFFECT OF HEAT AND PRESERVATIVES UPON THE PROPERTIES OF BACTERIAL ANTIGENS.

In preparing bacterial antigens the microörganisms are commonly killed by heating the suspensions at 60° C. to 100° C. for varying periods of time and various chemical germicides are added for preservation against contamination. The possible influences exerted by these two procedures upon the properties of bacterial antigens do not appear to have commanded much attention, but in these studies they have been found to possess some practical importance.

In other words, *heat and preservatives may* influence the hemolytic, anticomplementary and antigenic activities of bacterial antigens and any procedure resulting in reducing the first two and leaving the antigenic activity unaltered would enhance the practical value of an antigen and increase its antigenic index.

Methods Employed.—Antigens of simple saline suspensions have been prepared (approximately 2,000,000,000 per cc.) of living tubercle, typhoid, glanders and diphtheria bacilli and the gonococcus. Portions of each have been used living and after heating for one or two hours at temperatures varying from 55° C. to autoclaving at 5-pounds pressure. All were titrated for hemolytic, anticomplementary and antigenic activity.

The Effect of Heat Upon Bacterial Antigens.—Bacteria may be autoclaved before being used for the preparation of antigen as tubercle bacilli commonly are, or suspensions may be made of living microörganisms followed by heating in a water-bath at temperatures varying from 60° C. to 100° C. for varying periods of time.

All of the antigens employed were without hemolytic activity even in the maximum amounts, namely, 0.5 cc. of undiluted antigen, so that the influence of heating upon this property sometimes possessed by bacterial antigens cannot be expressed. It is well known however, that bacterial hemotoxins are thermolabile and it is highly probable that heating at 60° C. for one hour results in removing the hemolytic activity of bacterial antigens. Of course this has no bearing upon the hemolytic activity of the preservative; not infrequently the antigen itself is non-hemolytic until the preservative is added.

Heating tends to reduce the anticomplementary activity of bacteria antigens, that is, their power for non-specific fixation of complement. Almost invariably freshly prepared suspensions of living bacteria are more anticomplementary than portions of the same antigens heated for one or two hours at 60° C. or higher. Some antigens as those of *B. typhosus* and *B. diphtheriæ*, may not be influenced at 60° C., but the majority are and all have shown a slight reduction in anticomplementary activity after being heated at 80° C. or higher for one or two hours.

Heating has slight or no influence upon the antigenic activity of most bacterial antigens or their power for specific fixation of complement. Antigens of typhoid, tubercle and diphtheria bacilli were commonly unaffected even when suspensions were autoclaved for one hour. Some antigens of glanders bacilli were equally thermostabile while with others the antigenic activity was reduced when the suspensions were heated at 80° C. and higher; for example, one suspension was antigenic in dose of 0.5 cc. of 1 to 16 dilution unheated and after heating at 60° C. for two hours, but was reduced to 1 to 12 after heating at 80° C. for two hours and 1 to 10 after being boiled for two hours in an Arnold sterilizer or autoclaved at

5-pounds pressure. But heating some antigens in an Arnold for one or two hours or boiling with reflux condensers raises their antigenic activity which, along with a reduction of anticomplementary activity gives them higher antigenic indices.

The antigenic activities of gonococcus antigens were likewise sometimes affected by temperatures above 80° C., the power for specific fixation of complement being sometimes (not always) increased and in others slightly reduced when the suspensions were boiled or autoclaved for one to two hours.

The antigenic activities of typhoid bacilli antigens were also sometimes (not always) slightly reduced by heating at 80° C. or higher for one or two hours and but rarely increased, but antigens of tubercle bacilli were almost always slightly increased in antigenic activity by boiling for one or two hours.

Heating antigens at 80° C., boiling, or even autoclaving them for an hour or two may therefore increase their value in complement-fixation tests not only by slightly increasing their antigenic activities but also by reducing their anticomplementary activity and thereby producing higher antigenic indices as shown in Table 20. Indeed contrary to usual impressions it would appear that heating at 80° C. or higher for an hour or two improves the properties of practically all bacterial antigens. Certainly it does not reduce them as is likewise true of the Wassermann antigens. And their thermostability affords additional support for the hypothesis that the bacterial lipoids or lipoid-protein complexes are of importance in the mechanism of complement-fixation, exposure to high temperatures probably resulting in an improved dispersion of the antigenic complexes in the menstruum.

TABLE 20.—THE EFFECT OF HEAT UPON BACTERIAL ANTIGENS.

Methods.	Antigenic indices of antigens.				
	Typhoid.	Gonococcus.	Glanders.	Diphtheria.	Tuberculosis.
Unheated	5*	4	4	6	2.4
60° C. for two hours	5	4	4	6	3.0
80° C. for two hours	6	6	5	8	3.0
Arnold for two hours	6	7	8	8	4.0
Autoclave two hours (5 pounds)	6	7	8	8	4.0

* Means that the antigenic unit was five times less than the anticomplementary unit.

The Effect of Preservatives upon Bacterial Antigens.—Upon the completion of the preparation of saline or water antigens of bacteria it is customary to add a chemical antiseptic as a safeguard against

bacterial contamination. But the amount added is not a matter of indifference since the antiseptic may increase the hemolytic and anticomplementary effects of the antigen and thereby reduce its range or antigenic index.

Table 21 shows the hemolytic and anticomplementary effects of phenol, tricresol, formalin, and glycerin, the commonly employed antiseptics since the mercurials like bichloride are too hemolytic. These titrations were conducted in exactly the same manner as titrations of Wassermann antigens for hemolytic and anticomplementary activities; the table shows the highest dilutions in amounts of 0.5 cc. proving slightly hemolytic or slightly anticomplementary.

TABLE 21.—THE HEMOLYTIC AND ANTICOMPLEMENTARY ACTIVITY OF VARIOUS CHEMICAL ANTISEPTICS AND GERMICIDES.

Germicide.	Hemolytic activity, 0.5 cc. of:	Anticomplementary activity, 0.5 cc. of:
0.2 per cent phenol	−*	−†
0.5 per cent phenol	−	1:2
1.0 per cent phenol	−	1:3
2.0 per cent phenol	1:2	1:4
0.2 per cent tricresol	−	−
0.5 per cent tricresol	−	−
1.0 per cent tricresol	1:2	−
2.0 per cent tricresol	1:4	1:3
0.1 per cent formalin	−	1:2
0.5 per cent formalin	−	1:4
1.0 per cent formalin	−	1:8
2.0 per cent formalin	−	1:16
5.0 per cent glycerin	−	−
10.0 per cent glycerin	−	−
25.0 per cent glycerin	−	−
50.0 per cent glycerin	−	1:2

* Not hemolytic in 0.5 cc. of undiluted solution.
† Not anticomplementary in 0.5 cc. of undiluted solution.

Phenol was found to be more anticomplementary than tricresol but the latter was somewhat more hemolytic; formalin was free of hemolytic activity but decidedly more anticomplementary; glycerin was remarkably low in both hemolytic and anticomplementary activity although bacteriostasis requires approximately a 50 per cent cencentration which is slightly anticomplementary. A 25 per cent concentration of glycerin is antiseptic and without appreciable hemolytic and anticomplementary activity but I believe its use tends to increase the tendency to non-specific cross complement-fixation with syphilis antibody.

Similar results are also shown in Table 22 in which portions of the same antigen of *living* typhoid bacilli in saline solution were titrated for hemolytic, anticomplementary and antigenic activity with and without the addition of varying amounts of these preservatives.

TABLE 22.—THE EFFECT OF VARIOUS CHEMICAL GERMICIDES UPON AN ANTIGEN OF *B. typhosus*.

Preservatives.	Culture.	Hemolytic 0.5 cc. of:	Anticomplementary, 0.5 cc. of:	Antigenic 0.5 cc. of:	Antigenic indices.
None	+*	−†	1:2	1:32	16
0.2 per cent phenol	−	−	1:2	1:32	16
0.5 per cent phenol	−	−	1:4	1:32	8
1.0 per cent phenol	−	−	1:6	1:48	8
0.2 per cent tricresol	−	−	1:2	1:32	16
0.5 per cent tricresol	−	1:2	1:3	1:40	13
1.0 per cent tricresol	−	1:3	1:6	1:60	10
0.1 per cent formalin	−	−	1:4	1:32	8
0.5 per cent formalin	−	−	1:8	1:64	8
1.0 per cent formalin	−	−	1:20	1:40	2
10 per cent glycerin	+	−	1:2	1:32	16
25 per cent glycerin	+	−	1:4	1:32	8
50 per cent glycerin	+	−	1:6	1:42	7

* + = living bacilli; − = sterile.
† − = not hemolytic in 0. 5 cc. undiluted antigen.

Formalin in even 0.1 per cent concentration raised the anticomplementary unit and proved to be the least desirable of the four preservatives. Phenol and tricresol in 0.5 per cent raised the anticomplementary titers slightly, especially the former; glycerin in 25 per cent concentration had the same effect. Tricresol in 0.2 to 0.3 per cent is distinctly more bactericidal and antiseptic than similar concentrations of phenol and I believe constitutes the best preservative for bacterial antigens (saline or watery extracts) of the germicides in common use. Of course alcoholic antigens do not require the addition of these substances.

PRINCIPLES OF THE AUTHOR'S METHODS FOR PREPARING VARIOUS BACTERIAL ANTIGENS.

In my opinion the two outstanding conclusions from the large amount of investigation by others and myself upon bacterial antigens have been as follows: (*a*) that no one method stands out in a particular manner but (*b*) that in general terms those antigens are best, that is, have the longest range between anticomplementary and antigenic units (highest antigenic indices) which are composed of suspensions of the whole organisms in saline or suspensions after autolysis and disintegration. This is shown in Tables 23 and 24. Table 23 shows the *average* antigenic indices of antigens belonging to the six different groups as classified in this chapter. For the four organisms studied antigens of whole organisms in saline (Group II) and antigens of the dried and pulverized organisms suspended in

saline and boiled (Group IV) have given the longest ranges or highest indices. Table 24 shows the same results in a different manner observed by using each antigen in a dose of one-third of its anticomplementary unit and determining the highest dilution of its homologous immune serum in dose of 0.5 cc. giving ++++ reactions by my complement-fixation method described in Chapter XXXII.

The total or partial removal of the alcohol and ether-soluble lipoids from dried and pulverized organisms followed by the suspension of the bacterial residues in saline have generally been third best. Filtrates aiming to remove the bacterial fragments and retain the endotoxins (Group V antigens) have been generally inferior extracts.

TABLE 23.—THE AVERAGE ANTIGENIC INDICES OF BACTERIAL ANTIGENS PREPARED BY DIFFERENT METHODS.

Antigen groups.	Method of preparation.	Typhoid antigens.	Glanders antigens.	Tuberculosis antigens.	Gonococcus antigens.
I	Suspensions in culture medium	13*	Not tested	4.0	Not tested
II	Suspensions in saline	28	31	4.0	9.0
III	Autolyzed in water	22	27	2.7	8.4
IV	Dried and ground	41	49	4.0	10.0
V	Filtrates	3	12	2.2	4.0
VI	Dried and ground; lipoid extracted	38	30	3.4	10.0

* Means that the antigenic unit was thirteen times less than the anticomplementary unit.

TABLE 24.—HIGHEST AVERAGE DILUTIONS OF IMMUNE SERA IN DOSE OF 0.5 CC. GIVING STRONGLY POSITIVE COMPLEMENT-FIXATION REACTIONS WITH BACTERIAL ANTIGENS PREPARED BY DIFFERENT METHODS.

Antigen groups.	Method of preparation.	Typhoid serum (rabbit).	Glanders serum (horse).	Tuberculosis serum (calf).	Gonococcus serum (rabbit).
I	Suspensions in culture medium	1:70	Not tested	1:128	Not tested
II	Suspensions in saline	1:76	1:260	1:100	1:300
III	Autolyzed in water	1:43	1:230	1:95	1:240
IV	Dried and ground	1:190	1:420	1:160	1:260
V	Filtrates	1:10	1:240	1:60	1:160
VI	Dried and ground; lipoid extracted	1:154	1:140	1:78	1:145

Gonococcus complement-fixation tests conducted with the sera of 120 acute and chronic cases of gonorrhea in both sexes with seventeen different antigens by Dr. Charles deT. Shivers and myself have likewise yielded similar results as shown in Table 25, that is, antigens

of simple suspensions in saline or suspensions of the dried and ground organisms in saline followed by boiling for an hour or two, have generally proven best. This is in confirmation of the earlier results observed by Brown and myself[45] when simple suspensions of gonococci in saline solution were found to yield 11 per cent more true positive reactions in gonococcus infections than filtrate antigens.

TABLE 25.—PERCENTAGE OF POSITIVE GONOCOCCUS COMPLEMENT-FIXATION REACTIONS WITH ANTIGENS PREPARED BY DIFFERENT METHODS.

Antigen groups.	Method of preparation.	Positive reactions, per cent.
II	Suspensions in saline	27 to 40
III	Autolyzed in water	24 to 38
IV	Dried and ground	30 to 47
V	Filtrates	13 to 21
VI	Dried and ground; lipoid extracted	28 to 42

Antigens of Gonococci, Typhoid, Glanders, Contagious Abortion, etc. —These results have lead me to adopt the following two methods for the preparation of gonococcus, typhoid, paratyphoid, glanders, contagious abortion and all other bacterial antigens excepting those of tubercle bacilli but including those of molds and yeasts, the details of preparation being given in Chapters XXXII, XXXIII and XXXIV.

First Method.—The organisms are grown on a solid medium and removed with sterile distilled water to give a heavy suspension or they are grown in a fluid medium, removed by centrifuging and heavy suspensions prepared in water. Either is shaken to break up clumps, boiled with a reflux condenser or in an Arnold sterilizer for two hours, rendered isotonic by adding sodium chloride and preserved with 0.25 per cent tricresol or phenol. Titrate after standing in a refrigerator for a week or two.

Second Method.—The organisms are grown on a solid medium and removed with a minimum amount of sterile saline solution followed by centrifuging or are grown in a fluid medium and removed by centrifuging. The residues by either method are dried and pulverized and stored as such in ampoules. For use 0.1 gm. is suspended in 24 cc. of distilled water, boiled for two hours with a reflux condenser or in an Arnold sterilizer, rendered isotonic by the addition of 0.2 gm. sodium chloride and preserved with 1 cc. of 5 per cent tricresol or phenol. Titrate after standing in a refrigerator for a week or two.

When these antigens are used in amounts not greater than one-third or one-quarter of their anticomplementary units they do not ordinarily yield any cross complement-fixation reaction with syphilis antibody and therefore no attempt need be made to reduce or remove the lipoids from either Gram-positive or Gram-negative organisms.

Antigens of Tubercle and Other Acid-fast Bacilli.—But in the preparation of antigens of tubercle bacilli I believe it advisable at least to reduce the lipoids even though they may not be completely removed in order to limit as much as possible the chances of cross complement-fixation reactions with the sera of non-tuberculous syphilitic individuals. *In other words the problem in the preparation of antigen for the tuberculosis complement-fixation test is to produce one with a good antigenic index which does not give falsely positive or cross complement-fixation reactions with the sera of non-tuberculous syphilitic individuals when used in a dose sufficient for eliciting positive reactions with tuberculosis antibody in the sera of individuals with pulmonary tuberculosis.*

It is commonly stated that acid or fuchsin fastness is due to the lipoids and that extraction to the point of fuchsin negativity removes all or a part of these constituents. But the nature of acid-fastness is still an open question since long-continued extraction may fail to eliminate this quality completely and because so many dyes that are insoluble in fats stain the bacilli quickly and intensely. Furthermore, in my experience antigens prepared of fuchsin-negative bacilli are still capable of yielding a small percentage of falsely positive cross complement-fixation reactions with the sera of non-tuberculous but syphilitic individuals.

In the first place great difficulty has been experienced in rendering whole or dried and pulverized tubercle bacilli fuchsin-negative. Extractions with ether, alcohol, acetone, petroleum ether, benzol, toluol and carbon tetrachloride have failed at room temperature over periods up to twelve days. Ether, acetone, ethyl alcohol, methyl alcohol and benzol have also failed after as long as two hundred hours in Soxhlet extractions insofar as rendering *all* bacilli or fragments completely fuchsin-negative. Soxhlet extractions with ether for three days, followed by absolute ethyl alcohol for three days and lastly by acetone for three days have failed. Soxhlet extractions with petroleum ether have failed after eight hundred and seventy hours although benzol has occasionally extracted (Soxhlet) the lipoids to fuchsin negativity in about six hundred hours. I am, therefore, rather skeptical of the possibility of removing all lipoids from tubercle bacilli by these ordinary lipoid solvents if *complete* fuchsin negativity is the criterion. These methods, however, generally result in rendering most of the bacterial residues fuchsin-negative but antigens prepared by drying the residues, suspending 0.1 gm. amounts in 25 cc. of distilled water or 25 per cent solutions of glycerin in distilled water, boiling for an hour with a reflux condenser and rendering isotonic with sodium chloride, have generally yielded antigens of lower antigenic indices than control suspensions of dried bacilli before extraction although the extracted antigens also gave much less cross complement-fixation

with the sera of non-tuberculous but syphilitic individuals. In other words, extraction with lipoid solvents has had the tendency in my experience of reducing both specific complement-fixation in tuberculosis and of non-specific or cross fixation in syphilis and indicating, as previously mentioned when discussing the chemical nature of bacterial antigens, that the lipoids or lipoid-protein substances are antigenic; indeed as indicated by the investigations of Dienes and Scheff[46] a specific antigenic principle is alcohol soluble and its activity may be increased by the addition of commercial lecithin although as shown by Dienes and Freund[47] this alcohol-soluble substance is not the whole antigenic substance since the proteins and even a carbohydrate precipitable substance appeared to possess antigenic activity.

Antigens, prepared by Dreyer's method[48] in which certain lipoids soluble in acetone after treatment with formalin are removed, have yielded essentially similar results. In my experience the supernatant fluid antigens when titrated by my method have been less anticomplementary but also less antigenic with tuberculous sera than the antigens prepared of the bacillary residues but both have yielded cross complement-fixation reactions with the sera of non-tuberculous syphilitic individuals.

Even antigens prepared by suspending dried and pulverized tubercle bacilli in 0.1 to 1 per cent solutions of potassium hydroxide in absolute ethyl alcohol and extracting in a Soxhlet for ten to one hundred and twenty-five hours followed by recovery of the residues and the preparation of antigens of them by suspending 0.1 gm. amounts in 25 cc. of water followed by boiling for one to two hours, have yielded cross complement-fixation reactions with syphilitic sera although such antigens were but feebly hemolytic (0.5 cc. undiluted), anticomplementary in about 0.5 cc. of 1 to 8 and antigenic in 0.5 cc. of 1 to 24 to 1 to 90.

The Besredka broth culture antigen titrated for antigenic activity with a calf immune serum has given an antigenic index of 5; a similar antigen employing plain broth instead of Besredka's egg medium has given an index of 21; both yielded cross reactions with syphilitic sera. After extracting the Besredka broth antigen with ether the index was reduced to 2; extraction of the control plain broth antigen with ether reduced the index to 5; the former did not yield cross reactions with syphilitic sera while the latter yielded weak reactions. Antigens prepared by evaporating the ethers and dissolving the residues in alcohol gave indices of 5 and 8 respectively, indicating the antigenic activity of the ether-alcohol-soluble lipoids (probably largely the lecithins or acetone-insoluble lipoids); both yielded cross reactions with syphilitic sera. The residues obtained by acidulating Besredka broth cultures with glacial acetic acid, recovering the residues by centrifuging and suspending them in saline yielded

antigens with indices of approximately 3; similar antigens prepared of plain broth cultures gave indices of approximately 12; the former usually did not react with syphilitic sera while the latter usually yielded weak reactions. The supernatant fluids recovered after centrifuging the acidulated cultures and neutralizing gave indices of 4 and 6 respectively and both yielded cross reactions with syphilitic sera.

As previously stated, antigens prepared of suspensions of whole organisms in saline solutions or of suspensions of dried and pulverized organisms in saline followed by boiling for an hour or two have appeared to yield the longest range between anticomplementary and antigenic effects, that is, the highest antigenic indices. This has been generally true of tuberculosis antigens in my experience. In 1923 with the coöperation of Dr. Andrew Callahan I was able to apply complement-fixation tests to the sera of 112 patients from the wards of the Philadelphia General Hospital with each of 28 antigens prepared by the methods described in this chapter. Of these 66 to 81 per cent yielded positive reactions; 10 per cent yielded positive Wassermann reactions.

The suspension antigens of whole bacilli (Methods 2, 7 and 8) yielded from 44 to 80 per cent positive reactions; the antigens of dried, pulverized and boiled organisms (Methods 13, 22, 24, 25 and 26) yielded from 65 to 78 per cent positive reactions while the balance yielded from 3 to 60 per cent positive reactions. The glycerinated suspension of Petroff and Ornstein[49] yielded about 78 per cent positive reactions and was generally quite a satisfactory antigen.

Wassermann[50] has recently described a method for preparing antigen in which the lipoids, waxes and fats are removed from the bacilli with tetralin (tetra-hydrated nephthalin) as follows:

Well-grown human tubercle bacilli are removed from cerebral glycerin agar and about 2 gm. placed in an agate mortar along with sufficient pure tetralin to give a homogeneous paste. This paste is then suspended in about 200 cc. of tetralin along with glass beads and incubated at 32° C. in a shaking apparatus. The mixture is shaken daily for one to two hours until the bacilli are fuchsin-negative and changed into an amorphous mass approaching the blue contrast staining. The time required has varied considerably and with four to six weeks' old cultures has required from eight to twelve weeks. In many cases the tetralin had to be renewed in order to get rid of the last traces of red staining, when this is accomplished the deposit is centrifuged off, rinsed several times in ether to wash away the last traces of tetralin and the preparation placed in a desiccator. The resulting whitish-yellow powder is used, 0.1 gm. being suspended in 10 cc. of saline solution and titrated. I have had no experience with the antigen but the results reported by others are given in Chapter XXXII.

During the past three years I have used a suspension of bacilli from which a portion of the lipoids have been removed with good results, the principles of preparation being as follows (the details being given in Chapter XXXII):

1. The bacilli are cultivated in a glycerin-broth medium as in the preparation of tuberculin and the cultures autoclaved to kill the organisms.

2. The material is filtered on several layers of good paper and the residue washed free of glycerin.

3. The residue of bacteria is dried over sulphuric acid in a desiccator and then ground into a fine powder.

4. The powder is extracted with boiling ether, acetone and absolute ethyl alcohol, dried and stored.

5. For use 0.5 gm. of extracted powder is boiled for three hours with 48 cc. of distilled water, rendered isotonic by the addition of 0.5 gm. of sodium chloride and preserved with 2 cc. of 5 per cent tricresol or phenol. The resulting suspension antigen is kept in a refrigerator and used as required.

When titrated according to my method this antigen is usually not hemolytic in dose of 0.5 cc. undiluted, is anticomplementary in 0.5 cc. of approximately 1 to 2 and gives good fixation with a mixture of sera from non-syphilitic individuals with pulmonary tuberculosis in 0.5 cc. of approximately 1 to 30. About 3 antigenic units are employed in conducting the tests which corresponds to 0.5 cc. of 1 to 10, this dose being approximately one-fifth the anticomplementary unit. In this amount it only occasionally reacts with syphilitic sera containing large amounts of syphilis antibody and yielding strongly positive Wassermann reactions. This seems unavoidable since the more complete removal of the ether-alcohol-soluble lipoids reduces antigenic activity with tuberculosis antibody. Therefore, I believe that the Wassermann test should be conducted routinely with all sera submitted for the tuberculosis complement-fixation test. If both yield positive reactions due care must be exercised in the interpretation of the latter.

THE KEEPING QUALITIES OF BACTERIAL ANTIGENS.

It is commonly stated that saline and watery antigens of bacteria do not keep well and that they may suddenly and unaccountably undergo changes in anticomplementary and antigenic activity. For many years I shared this opinion but during the past four years have found it necessary to revise these notions on the basis of larger experience. In a general way it is true that saline extracts are more likely to change than alcoholic extracts but if the former are preserved with 0.2 to 0.3 per cent of tricresol or phenol as a precaution against contamination and if the extracts are kept reasonably cold

as at 4° to 8° C. deterioration occurs very slowly and extracts may be kept practically unchanged for many months and even a year or two. At least this has been found true of the antigens prepared by my methods outlined in this chapter. Indeed during the past several years I have given up the idea that a bacterial antigen requires titration, at least for anticomplementary activity, prior to each set of complement-fixation tests. I have found it quite sufficient to titrate only every two or three months as is advisable in the case of the antigen for the syphilis complement-fixation test and even this practice is not absolutely necessary. If bacterial antigens are allowed to ripen for two or three weeks in a refrigerator before the titrations are conducted it is usual for the hemolytic, anticomplementary and antigenic titers to remain practically unchanged for six months or longer. Even at room temperature I have found antigens of gonococci, typhoid and glanders bacilli to keep unchanged in anticomplementary and antigenic properties for periods up to four to six months although preservative in a refrigerator is to be preferred. Saline dilutions of these antigens have also been kept in a refrigerator unchanged for months at a time although I believe it better to dilute only sufficient of the stock antigen for each day's work.

Dried and pulverized bacterial residues of tubercle, typhoid and glanders bacilli and gonococci prepared according to the second method outlined above, have been kept in sealed ampoules at room temperature for several years without change in properties.

Miss Trist and I have also saturated filter papers with heavy saline suspensions of these antigens after Noguchi's method of preserving hemolysin and antigen for his syphilis test (about 2.5 cc. to each paper 12 x 12 c.mm.) and after drying have kept them in tightly stoppered vials in a refrigerator for periods up to a year without change in their anticomplementary properties; the majority however, lost in antigenic sensitiveness during the first two weeks although a part of this property was retained for several months.

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CHAPTER XVI.

ANTIGENS FOR COMPLEMENT-FIXATION TESTS IN PROTOZOAL AND METAZOAL DISEASES AND FOR THE DIFFERENTIATION OF PROTEINS.

Complement-fixation tests have had only a limited application in diseases caused by protozoa and metazoa. But as stated in Chapter XLIX investigations have indicated that some complement-fixing antibody may be produced in trypanosomiasis, intestinal helminthiasis and especially when helminths have invaded the tissues of the host as in echinococcosis, bilharziosis and liver fluke infestations.

NATURE OF HELMINTHIC ANTIGENS.

Antigens for complement-fixation tests in these diseases have been prepared by extracting the parasites with saline solution and with alcohol and the question has been raised whether essential reacting substances are proteins, lipoids or protein-lipoid complexes. Ghedini[1] has employed saline extracts of hookworms; likewise Weinberg and Parvu[2] in the preparation of antigens of tapeworms and ascarides. Trist, Heist and myself[3] used both saline and alcoholic extracts of tapeworms, ascarides and whip-worms in a study of complement-fixation in helminthiasis of dogs; both yielded positive reactions, the alcoholic extracts being somewhat superior to the saline ones. Both, and especially the alcoholic antigens, contained enough lipoidal material to give Wassermann reactions with the sera of syphilitic subjects.

Parvu[4] has employed alcoholic extracts of hydatid fluid as likewise Israel[5] who regarded hydatid antigen as essentially lipoidal. Fairley[6] has been especially interested in this subject and found that alcoholic extracts of the scolices yielded the best antigen, extracts of the cyst walls and of the fluid being practically non-antigenic. These observations have been confirmed by Patterson and Williams.[7]

Weinberg[8] has also demonstrated complement-fixation in liver fluke infestations with saline extracts of the flukes. Sevantie[9] and Fairley and Williams[10] have employed alcoholic extracts with success. Yoshimoto[11] and Sueyasu[12] have reported positive complement-fixation reactions in infestations with *Schistosomum japonicum* employing alcoholic extracts of the adult worms. Fairley[13] failed

to obtain satisfactory antigens with adult bilharzia worms but evolved a test for detecting *S. mansoni* and *S. hematobium* infestations in man and experimentally infested monkeys by extracting with absolute alcohol the bilharzia cercariæ located in the livers of infested intermediate snails (*Planorbis boissyi* and *Bullinus dybowski*). Murray[14] confirmed the value of the antigen and the specificity of the test. Le Bas[15] has stated that the antigen could not be regarded as of a lipoidal nature but rather a protein substance soluble in 50 per cent alcohol; the acetone-soluble lipoids were reported as devoid of antigenic properties.

Fairley,[16] however, insists that the reacting substance in alcoholic extracts of both moist fresh and dried livers of snails is a lipoid or a lipoidal complex of colloidal nature and extractions with alcohol calculated at 96.65 per cent failed to reveal the presence of protein by the biuret, Hopkins-Cole, ninhydrin and Millon color tests for protein. Indeed he has reported[17] that these alcoholic cercarial extracts are not only antigenic in the test-tube but likewise true antigens capable of engendering the production of specific antibody when injected into goats. Fairley[18] has also reported positive reactions with his alcoholic extracts in tests employing the sera of goats artificially infested with *Schistosomum spindalis;* group reactions occurred with *Schistosomum indicum* and *Schistosomum hematobium* but outside the mammalian bilharzia group specificity was absolute, sera from no other helminthic infestation yielding pseudo-reactions.

It would appear, therefore, that the situation in relation to the chemical nature of the antigens for complement-fixation tests in protozoal and metazoal diseases is somewhat similar to that in bacterial antigens namely, that while hitherto the proteins have been regarded as the active constituents, the lipoids may be antigenically active in a manner analogous to the role of tissue lipoids in the extracts employed as antigens in the Wassermann reaction. There is, however, one important point of difference, namely, while the syphilis reaction is usually conducted with lipoids or lipoid-protein complexes from non-syphilitic tissues, the extracts of bacteria, protozoa and metazoa must be prepared from the organisms themselves; in other words, the latter are specific, suggesting the role of protein in association with the lipoids. On the other hand, the matter cannot be regarded as a closed subject but a fit one for further chemical research. Insofar as the production of antibody in animals is concerned I believe it will be ultimately proven that a trace of protein at least must be present in the antigen along with the lipoidal constituents of the parasites; in the test-tube the lipoids would certainly appear to be antigenically active although even here a trace of protein may be required; that is, lipoid-protein complexes may constitute the antigenic substance both *in vivo* and *in vitro*.

Be that as it may, and the question demands additional chemical investigation for final elucidation, both saline and alcoholic extracts of helminths may be employed as antigens in complement-fixation tests and in Chapter XXXIV detailed directions for their preparation are given.

Furthermore both saline and alcoholic extracts of animal parasites usually contain sufficient lipoids to react with syphilis antibody. In complement-fixation tests for infestments among the lower animals this is of no consequence but among human subjects it is very important and especially in the serum diagnosis of echinococcus disease. For this reason I believe it advisable to remove some of the alcohol and ether-soluble lipoids from the wet or dried parasite material before preparing an antigen and my methods, fashioned after those for preparing antigens of tubercle bacilli, include preliminary ether and alcohol extractions. Extracts of the residues are still highly antigenic and less likely to yield cross complement-fixation reactions with syphilis antibody.

ANTIGENS FOR THE DIFFERENTIATION OF PROTEINS.

Under this heading I refer to antigens of serum, blood, semen, milk, meats, etc., for purposes of identification and differentiation by the complement-fixation reaction. Antigens for the identification and differentiation of bacteria have been already discussed in the preceding chapter.

It may be that the lipoids or lipoid-protein complexes of serum, blood, semen, meats, etc., constitute the active antigenic substances *in vitro* but it would appear to be the consensus of opinion that *the proteins are more actively concerned.*

In order to obtain some information upon this subject Miss Rule, Mr. Brown and I have prepared saline, alcoholic and acetone-insoluble lipoid extracts of fresh muscle from sheep, hogs, dogs and cattle. Wet tissues were used, the saline extracts being prepared by extracting 10 gm. of finely minced tissue with 100 cc. of phenolized saline solution at 37° C. for ten days. The alcoholic extracts were prepared by extracting the same amount of tissue with absolute ethyl alcohol for the same time, the tissue juices reducing the concentration of alcohol to 96 to 97 per cent. The extracts of acetone-insoluble lipoids were prepared by extracting 10 gm. of tissue with absolute ethyl alcohol, evaporating, extracting the residues with ether and precipitating the lipoids with an excess of acetone according to Noguchi's method for preparing antigen for the Wassermann test. Each extract was tested for proteins and titrated for anti-complementary and antigenic activity, the latter with homologous rabbit immune sera. The results are summarized in Table 26 and show that the saline and alcoholic extracts were antigenic as expected

and especially the saline extracts because they contained soluble proteins and lipoids in colloidal suspension while the latter contained lipoids and heavy traces of protein. Most interest, however, centered in the acetone-insoluble lipoid extracts because they gave only faintly positive protein reactions but were, nevertheless, distinctly antigenic *in vitro*.

TABLE 26.—THE PROPERTIES OF SALINE, ALCOHOLIC AND ACETONE-INSOLUBLE LIPOID ANTIGENS OF MUSCLE.

Antigens of.	Anticomplementary units.			Antigenic units.			Antigenic indices.		
	Saline 0.5 cc. of:	Alcoholic 0.5 cc. of:	Acetone insol. lipoids 0.5 cc. of:	Saline 0.5 cc. of:	Alcoholic 0.5 cc. of:	Acetone insol. lipoids 0.5 cc. of:	Saline 0.5 cc. of:	Alcoholic 0.5 cc. of:	Acetone insol. lipoids 0.5 cc. of:
Sheep muscle . .	1:2	1:4	1:2	1:12	1:16	1:10	6	3	6
Hog muscle . . .	1:2	1:4	1:2	1:8	1:12	1:12	4	2	4
Dog muscle . . .	1:2	1:16	1:4	1:8	1:64	1:16	4	4	4
Beef muscle . .	1:2	1:4	1:2	1:32	1:8	1:8	16	2	4

It would appear therefore, that the situation in respect to these antigens is similar to that in relation to the antigens of bacteria, protozoa and metazoa, namely, that the lipoids or lipoid-protein complexes are actively antigenic *in vitro* even though they may be inactive or less antigenic *in vivo* in producing antibody.

Of course, the lipoids of all of these extracts yield positive Wassermann reactions with the sera of some syphilitic individuals but this is of no consequence in using them for the identification and differentiation of meats since the immune sera employed (usually rabbit immune sera) do not contain syphilis antibody. Therefore, in the methods described in Chapter XXXVI for their preparation no effort is made to reduce or remove the lipoids.

Antigens of blood serum, semen, milk and such substances are usually saline dilutions. Blood stains are likewise usually saline extracts prepared in much the same manner as for the precipitation tests. Antigens of fresh blood are usually prepared with distilled water to produce complete hemolysis followed by the addition of 0.8 gm. of sodium chloride to each 100 cc. to restore isotonicity. The precipitated and purified proteins of these substances may be employed but without any particular advantage. In my experience these saline or watery extracts are practically always more antigenic in complement-fixation tests than alcoholic extracts or extracts of acetone-insoluble lipoids. The results of one experiment for example are shown in Table 27 where a watery extract of dried human blood gave an antigenic index of 64 when titrated with rabbit antihuman serum whereas the indices obtained with alcoholic (absolute ethyl)

and acetone-insoluble lipoid extracts of the same material were but 2.

TABLE 27.—THE PROPERTIES OF SALINE, ALCOHOLIC AND ACETONE-INSOLUBLE LIPOID ANTIGENS OF HUMAN BLOOD.

Extracts.	Anticomplementary units.	Antigenic units.	Antigenic indices.
Saline	0.5 cc. of 1:2	0.5 cc. of 1:128	64
Alcoholic	0.5 cc. of 1:2	0.5 cc. of 1:4	2
Acetone-insoluble lipoids .	0.5 cc. of 1:2	0.5 cc. of 1:4	2

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CHAPTER XVII.

METHOD OF DILUTION AND TITRATION OF ANTIGENS AND THE AMOUNTS TO EMPLOY IN COMPLEMENT-FIXATION TESTS WITH SPECIAL REFERENCE TO THE KOLMER METHODS.

The best of antigens for complement-fixation tests for syphilis and other diseases may yield poor results if employed in the wrong amounts. On the other hand, a relatively poor antigen may be made to yield fairly good results if it happens to be used in the best or optimum amount. Any antigen in too large a dose may give either falsely positive or falsely negative reactions and this is especially true of those employed in the Wassermann test; any used in too small an amount may readily give falsely negative reactions. Therefore the titration of antigens and the determination of the best or optimum amounts to use for eliciting the most sensitive and specific reaction constitutes one of the most important phases of complement-fixation technique.

PROPERTIES OF ANTIGENS IN RELATION TO TITRATIONS.

As stated in preceding chapters every antigen for use in complement-fixation tests possesses some degree of *anticomplementary action.* This means that in certain amounts it is capable *itself* and *without the presence of antibody* of absorbing, fixing or inactivating a portion or all of the complement. Needless to state if an antigen were employed in this amount in the Wassermann or other complement-fixation tests all of the reactions would show a partial or complete inhibition of hemolysis which may be erroneously interpreted as positive reactions. Therefore one of the first essentials is to determine the *anticomplementary unit* of an antigen, which is the smallest amount capable of fixing or absorbing even a portion of complement in a non-specific manner, that is, in the absence of antibody.

As a general rule the extract also possesses *antigenic activity,* that is, the property of specifically fixing or inactivating complement in the presence of antibody. The smallest amount yielding a strongly positive reaction is designated as the *unit* and the aim is to prepare an antigen with the minimum of anticomplementary and the maximum of antigenic activity, that is, one with the longest range or best antigenic index.

Some antigens are also *hemolytic*, that is, capable of hemolyzing corpuscles themselves just as an acid or alkali may destroy these cells. Alcoholic tissue extracts employed in the Wassermann test must be especially watched for this property; likewise alcoholic extracts of bacteria and animal parasites; saline extracts are much less likely to be hemolytic. Obviously if an extract were employed in a hemolytic dose it is likely that the corpuscles would be partially or completely hemolyzed with an end-result resembling a negative complement-fixation reaction. Therefore the smallest amount of antigen itself capable of hemolyzing corpuscles is designated as the *hemolytic unit* but this is almost always an amount larger than the anticomplementary unit; for this reason it is not usually necessary to determine the hemolytic unit if the anticomplementary unit has been ascertained.

FACTORS INFLUENCING THE ANTICOMPLEMENTARY TITRATION OF ANTIGENS.

The Influence of Primary Incubation.—Some serologists do not incubate the antigen-complement mixtures before adding hemolysin and corpuscles to determine the degree of complement-fixation or destruction; others incubate a variable period not at all similar to the primary incubation employed in their complement-fixation tests and others use one-half or one hour in a water-bath or air incubator and conduct the main complement-fixation tests with a primary incubation in a refrigerator. These practices introduce error; *in conducting this titration the antigen-complement mixtures should receive exactly the same primary incubation as employed in the* complement-fixation tests. For example, an antigen was free of anticomplementary effects in 1 cc. of 1 to 10 dilution when hemolysin and cells were added at once but with a primary incubation of one hour in a water-bath the unit was 0.6 cc. and with eighteen hours in a refrigerator at 8° C. the unit was 0.2 cc., indicating the important relation of primary incubation to anticomplementary titration and especially since antigens are more anticomplementary after refrigerator incubation than one hour in a water-bath or air-incubator. For this reason a complement-fixation technique based upon water-bath incubation may not be adapted for the refrigerator method; *certainly a dose of antigen based upon water-bath incubation may be too large for complement-fixation tests conducted with a primary incubation in a refrigerator for eighteen hours.*

The Influence of Method of Diluting Antigens with Saline Solution.—Sachs and Roudini,[1] Browning and McKenzie[2] and others claim that Wassermann antigens *slowly* diluted with saline solution are more turbid and antigenic than opalescent emulsions prepared by

mixing antigen and saline rapidly; less has been written on the influence exerted upon anticomplementary effects. Bronfenbrenner and Schlesinger[3] found that opaque emulsions of acetone-insoluble lipoids are more anticomplementary and the fluorescent are more antigenic; Ruediger[4] has also found that the degree of turbidity and dilution of alcoholic extracts greatly influences the results of titrations and that for each antigen there appears to be an optimum turbidity and dilution.

In the experiments of Miss Trist and myself[5] four emulsions were prepared of each of three antigens as follows:

1. Saline was added to each extract at the rate of 1 cc. per minute; these emulsions were very opaque or turbid.

2. Saline was added to each extract very rapidly by blowing from a 5 or 10 cc. pipette; these emulsions were opalescent.

3. Extract was added to saline drop by drop from a 1 cc. pipette; these emulsions were opaque.

4. Extract was added to saline very rapidly by blowing from a 1 cc. pipette; these emulsions were opalescent.

The results of titrations of these four emulsions of each of three antigens conducted at the same time with a primary incubation of eighteen hours at 8° C. as well as similar titrations of eight additional extracts diluted in the four ways with a primary incubation of one hour in a water-bath, have given the following results:

1. *The differences were usually slight but occasionally definite and especially with cholesterolized extracts.*

2. In the majority of titrations the turbid or opaque emulsions prepared by adding saline very slowly to the extract or the extract very slowly to the saline were more anticomplementary than the opalescent emulsions prepared by adding saline or extract rapidly.

3. Opalescent emulsions prepared by adding the saline rapidly to the extract were somewhat more anticomplementary than emulsions by adding the extract rapidly to the saline.

4. Turbid or opaque emulsions prepared by adding the saline slowly to the extract were generally of the same anticomplementary activity as emulsions prepared by adding the extract slowly to the saline.

Bordet's[6] method of diluting antigen consists of evaporating 1 part to dryness. The dried residuum is then suspended in $2\frac{1}{2}$ parts of distilled water, the first cubic centimeter being added slowly with a 0.2 cc. pipette and mixed as thoroughly as possible after the addition of each 0.2 cc. The remainder of the water may be added rapidly, but the suspension must be thoroughly mixed. The required amount of this suspension is then added to the amount of saline solution necessary to give the desired dilution, which is mixed well by shaking. Miss Yagle and I have found that the suspensions are quite turbid and the average anticomplementary unit of my antigen

(cholesterolized and lecithinized alcoholic extract of beef heart) was 0.5 cc. of 1 to 5 dilution whereas the unit for the same extracts diluted by adding the antigen to saline or saline to antigen was 0.5 cc. of 1 to 8 to 1 to 10.

From the standpoint of minimum anticomplementary activity opalescent antigens are preferable to opaque or turbid antigens for the Wassermann test and especially opalescent emulsions prepared by slowly adding the extract to the saline solution; this also results in giving the maximum degree of antigenic activity as will be shortly discussed.

The same is true of alcoholic antigens of bacteria, protozoa and metazoa but with saline extracts of tissues and bacteria I have never been able to elicit differences worthy of mention probably because the dispersion of colloids is not influenced to the same degree as diluting alcoholic extracts with saline solution.

The Influence of Normal Serum.—Probably the majority of serologists omit serum in titrations for the anticomplementary activity of antigens. The presence of normal serum however, usually has an influence upon the results when an antisheep hemolytic system is employed, due to the presence of natural hemolysins. When antigens are titrated in the presence of 0.1 cc. heated normal serum the units of anticomplementary activity are usually slightly less than when the antigens are titrated without the addition of serum. When the titrations are conducted with serum heated for fifteen minutes followed by removal of hemolysins by absorption with sheep corpuscles and reheated for fifteen minutes to remove anticomplementary activity, antigens are sometimes more anticomplementary than when titrated plain or in the presence of unabsorbed serum; these results were probably due to a slight anticomplementary activity of the serum, although the serum controls may show complete hemolysis.

By reason of the influence of natural hemolysins and possibly of anticomplementary activity of serum upon the results of antigen titrations, the amount of serum to employ becomes a matter of practical importance. In my quantitative complement-fixation test each serum is tested in five amounts ranging from 0.1 to 0.0025 cc.; experience has shown that titrations of antigens conducted with 0.05 cc. of *a mixture of fresh normal sera heated at 55° C. for fifteen minutes* yield uniform and satisfactory results.

Theoretically serum should always be used in the anticomplementary titrations of antigens although practically this does not appear absolutely necessary; if serum is used it must be perfectly fresh, heated in the same manner as are the sera for complement-fixation tests, used in an average amount and preferably as mixed sera from which natural antisheep hemolysin has been removed. In the titration of antigens for the Wassermann test it is not difficult to supply normal serum but in the titration of bacterial antigens and especially those of the

tubercle bacillus this may be more difficult; therefore in titrating the latter serum is generally omitted.

The Removal of Anticomplementary Substances.—A few serologists are of the opinion that heating saline emulsions of Wassermann antigens in a water-bath at 55° C. for one-half hour or more, results in a reduction of anticomplementary activity; this may be true to a slight degree with emulsions kept over from day to day and contaminated with bacteria, but with freshly prepared emulsions the results of experiments of Trist and myself have been uniformly negative; likewise with alcoholic and saline antigens of bacteria.

After boiling undiluted alcoholic extracts for one hour with reflux condensers the anticomplementary activities appeared to be very slightly reduced with some extracts, likewise with some bacterial antigens; but *the influence of heat upon the anticomplementary activities of alcoholic tissue extracts and bacterial antigens is so slight, however, as to be negligible.*

Filtration of saline emulsions of alcoholic tissue extracts through chemically clean and sterile Kitasato earthen candles completely removes the anticomplementary substances and this would be an ideal method for removing them and those producing hemolysis, were it not for the fact that the antigenic substances are totally removed at the same time.

Sachs has described a useful method for removing anticomplementary substances from serum by adding 8.2 cc. of $\frac{N}{300}$ hydrochloric acid to 1 cc.. standing one-half hour, centrifuging and using the supernatant fluid to which is added 0.8 cc. of 10 per cent sodium chloride solution. Miss Rule and I have applied this method to bacterial antigens but in all instances the supernatant fluids were likewise rendered entirely or almost free of antigenic substances as well. After an antigen has been finished I know of no way of removing or materially reducing the objectionable anticomplementary substances without equal removal of the antigenic constituents.

FACTORS INFLUENCING ANTIGENIC TITRATIONS.

The Influence of Primary Incubation.—As stated in Chapters XXIII, XXIV and XXV, temperature and duration of primary incubation exert a powerful influence upon the antigenic activity of *all* antigens. With undiluted serum containing large amounts of antibody, fixation is a rapid phenomenon and may occur in a few minutes at room temperature, but with sera containing smaller amounts of antibody and with dilutions of sera, longer periods of time are required for maximum fixation.

The stronger reactions observed after a primary incubation of antigen, antibody and complement at 8° C. for eighteen hours as compared with one hour at 37° C. have been ascribed to a greater

deterioration of complement, greater non-specific fixation, and greater specific fixation by antibody and extract. For these reasons *the primary incubation of antigen, antibody serum and complement in the titration of antigen should be exactly the same as used in the main complement-fixation tests.*

The Influence of Method of Diluting Extracts with Saline Solution.— Numerous experiments with different extracts have been conducted on this important subject; as previously described, four different emulsions were prepared with each extract. Each was titrated for the antigenic unit with two kinds of primary incubation, one hour at 37° C. and eighteen hours at 8° C. and the results may be summarized as follows:

As a general rule turbid emulsions of alcoholic tissue extracts prepared by adding saline very slowly to extracts or extracts very slowly to saline, were slightly more antigenic than opalescent emulsions prepared by rapid mixture of extracts and saline.

Emulsions prepared by slowly adding extract to saline were not as turbid as emulsions prepared by slowly adding saline to extract; the former were opalescent rather than turbid but equally antigenic. Since the latter were less anticomplementary I have concluded that *antigens for the Wassermann test are best diluted for complement-fixation tests by slowly adding the extract to the saline and shaking gently after each addition.*

The method of diluting antigen by the Bordet method is given on page 243; as a general rule the suspensions are turbid and may show some coarse aggregates. In a comparative study by Miss Yagle and me of this method and my own consisting of adding the extract drop by drop to the required amount of saline and shaking after each addition, it was found with my cholesterolized and lecithinized alcoholic extracts of beef heart that the latter method usually yielded the higher antigenic units but curiously enough the same antigens diluted by Bordet's method sometimes yielded stronger complement-fixation reactions in routine work. Miss Moore[1] I believe has observed the same curious phenomenon. For example, the *antigenic units* of three different antigens diluted by the two methods and titrated at the same time have been as follows:

	Antigen No. 1.	Antigen No. 2.	Antigen No. 3.
Diluted by Bordet method	0.5 cc. 1:1200	0.5 cc. 1:1200	0.5 cc. 1:600
Diluted by Kolmer method	0.5 cc. 1:3200	0.5 cc. 0:3200	0.5 cc. 1:1600

Antigen No. 1 used in a dose of 10 antigenic units (0.5 cc. of 1 to 320) frequently gave stronger reactions when diluted according to Bordet's method than when diluted by my method and in one series of comparative tests gave approximately 3 per cent more true positive reactions. However, in this series the Bordet dilutions also gave about 5 per cent weak falsely positive reactions with sera from non-syphilitic individuals.

In a second series of comparative tests with 83 sera from syphilitic and non-syphilitic individuals antigen diluted by Bordet's method gave positive reactions with 25 or approximately 30 per cent; but 3 of these sera were from non-syphilitic individuals and the reactions non-specific. Practically the same results were observed when the antigen was diluted by adding saline to extract. The antigen diluted by my method (extract added to saline) gave positive reactions with 22 sera or about 26.5 per cent but all were specific. In other words *my antigen diluted by Bordet's method undoubtedly gives many more strong reactions than when diluted by my method but this advantage is offset by a small percentage of apparently non-specific reactions.* For a while I was on the point of adopting Bordet's technique but when I became convinced that it tended to render cholesterolized extracts too sensitive I decided to adhere to my own method in an attempt to render the test as sensitive as possible with specificity. The result have shown, therefore, that the method of diluting antigen is of considerable importance in relation to the Wassermann reaction and especially by reason of the influence exerted upon the antigenic activity of extracts.

The same has been found true of alcoholic extracts of bacterial and especially of the tubercle bacillus; likewise in the case of alcoholic extracts of tapeworms, echinococcus scolices, etc., but with watery or saline antigens of tissues and bacteria as well as of blood and serum the method of dilution with saline solution has had no appreciable effect probably because without influence upon the dispersion of lipoid-protein complexes in such preparations.

The Relation of Serum.—The amount of syphilitic serum to employ in the titration of syphilis antigens is a matter of considerable importance; also the strength of the serum in syphilitic antibodies. Most serologists use 0.1 or 0.2 cc. of strongly positive serum; others use similar quantities of moderately positive serum.

A large number of experiments have been conducted on this phase of the problem with the following conclusions:

1. A mixture of several syphilitic sera is preferable to the use of a serum from a single individual.

2. Sera yielding strongly positive reactions are to be preferred because the unit is better defined.

3. If one amount of serum as 0.1 cc. is being employed in the main tests a similar amount should be used in the antigen titrations; if the main tests are being conducted with varying amounts of serum the average should be used and in my method 0.05 cc. (0.5 cc. of 1 to 10) is employed.

4. The sera should be free of anticomplementary activity and natural hemolysin and heated in exactly the same manner as in the main complement-fixation tests.

A common mistake in titrating bacterial antigens (notably of the

gonococcus) for complement-fixation tests with human serum is to use immune horse, goat or some other animal serum instead of human serum. *The horse or other immune serum may contain so much antibody that the antigenic unit of the antigen is a correspondingly high dilution and the employment of 2 units or even 10 units may be insufficient for complement-fixation tests with human sera containing much less antibody.*

When antigens are titrated with these highly immune sera the antigenic units may be 0.5 cc. of dilutions as high as 1 to 100 or even 1 to 300 so that 10 units or 0.5 cc. of 1 to 10 or 1 to 30 may represent a dose of five times or more less than the anticomplementary unit, *but too small an amount for tests employing human sera.* The following examples have been selected from a large series:

(*A*) The anticomplementary unit of a gonococcus antigen was 0.5 cc. of 1 to 2; with 0.05 cc. of a goat immune serum the antigenic unit was 0.5 cc. of 1 to 64 (antigenic index 32); with 0.1 cc. of a mixture of 3 positive human sera the antigenic unit was 0.5 cc. of 1 to 8 (antigenic index 4). Two antigenic units or even 5 units as based upon the results of titration with the goat immune serum were, therefore, too small amounts of antigen for the technique of this gonococcus complement-fixation test employing human sera.

(*B*) The anticomplementary unit of a tuberculosis antigen was 0.5 cc. of a 1 to 4 dilution. The antigenic unit with 0.05 cc. of a calf immune serum was 0.5 cc. of 1 to 160 (antigenic index 40); with 0.1 cc. of a mixture of positive human sera the antigenic unit was 0.5 cc. of 1 to 16 (antigenic index 4). Here again the general rule of employing 2 antigenic units based upon titration with the goat immune serum fell far short of sufficient antigen for tests with human sera.

(*C*) The anticomplementary unit of a glanders antigen was 0.5 cc. of 1 to 2. The antigenic unit with 0.005 cc. of a highly immune rabbit serum was 0.5 cc. of 1 to 120 (antigenic index 60). The antigenic unit with 0.1 cc. of a mixture of sera from glanderous horses was 0.5 cc. of 1 to 10 (antigenic index 5). A dose of 10 units based upon the results of titration with the rabbit immune serum was 0.5 cc. of 1 to 12 but this amount was too small for securing positive reactions with average sera from glanderous horses.

Immune sera of the lower animals may be employed for determining the antigenic sensitiveness of various antigens but if so, the amount employed in the titrations should carry approximately the same amount of antibody as contained in 0.05 cc. of average human serum being submitted to the complement-fixation test. This will avoid the possibility of using antigens defective in antigenic sensitiveness for routine complement-fixation tests. In other words, simply because an antigen proves satisfactory when titrated with 0.1 or 0.05 cc. of a highly artificially immune serum is not sufficient;

it must prove antigenic when titrated with 0.1 or 0.05 cc. of human serum or 0.1 or 0.05 cc. of such a *dilution* of artificial immune serum as will carry an amount of antibody approximately equal to the average amount contained in 0.1 or 0.05 cc. of human serum or that of infected lower animal (as, for example, glanderous horses when titrating antigen for the glanders test).

The Influence of Heat Upon the Antigenic Properties of Antigens.—Heating saline dilutions of various alcoholic tissue extracts employed as antigens in the Wassermann test with reflux condensers does not regularly alter their antigenic activity. Heating at 56° C. for thirty to sixty minutes has had no appreciable effect in my experience. At 62° and 100° C. for one to two hours the antigenic activity of some extracts is slightly increased but much more frequently there occurs a slight decrease in antigenic activity. The same results have been observed with alcoholic and saline extracts of bacteria so that while boiling may aid in the preparation of antigens and especially of bacteria as discussed in the preceding chapter, three does not appear to be any advantage in heating the saline dilutions of antigens from the standpoint of materially reducing their anti-complementary activity or increasing their antigenic effects.

FACTORS INFLUENCING THE HEMOLYTIC TITRATION.

The Influence of Incubation.—Heat greatly favors the hemolytic activity of tissue extracts; all extracts employed in our study were more hemolytic at 37° C. in a water-bath for one hour than in a refrigerator at 8° C. for eighteen hours. Plain or crude alcoholic extracts were usually most hemolytic; the addition of cholesterol usually reduced hemolytic activity due probably to the anti-hemolytic activity of this substance. Acetone-insoluble lipoids and my cholesterolized lecithinized alcoholic extracts of beef heart proved least hemolytic. The manner of diluting alcoholic extracts with saline solution does not appear, however, to influence hemolytic activity.

The Influence of Serum.—Most serologists titrate the hemolytic activity of an extract by placing increasing amounts of an emulsion in test-tubes with the corpuscles followed by incubation; as shown in our experiments however, *the presence of serum greatly reduces hemolytic activity and should always be used in the titration in order to test the extract under the same conditions as it is used in complement-fixation tests.* Either guinea-pig or human serum (syphilitic or non-syphilitic) may be used; in my method 0.5 cc. of 1 to 10 human serum is employed.

The Influence of Heat.—Heating saline emulsions of alcoholic extracts in a water-bath at 55° C. for one-half hour appeared to slightly reduce hemolytic activities; likewise boiling alcoholic

extracts for one hour with reflux condensers appeared to reduce the hemolytic activity of some extracts to a slight extent but the influence of heat is so slight that the procedure is not of practical value.

The Influence of Filtration.—Filtration of saline emulsions of various extracts through chemically clean and sterile Kitasato filters completely removes the hemolytic substances; however, this procedure also removes the antigenic substances and is worthless as a practical procedure unless further studies show that the use of filters of varying density results in the removal of hemolytic and anticomplementary substances and not of antigenic substances.

THE OPTIMUM AMOUNT OF ANTIGEN TO EMPLOY IN COMPLEMENT-FIXATION TESTS.

The amount of antigen employed in complement-fixation tests in syphilis and other diseases is equally important as the kind of antigen; different methods are in use for determining the amount of antigen to employ and these as well as the use of different kinds of antigen are largely responsible for the variation in specificity and sensitiveness of complement-fixation tests in different laboratories.

Some serologists apparently do not hesitate to use an antigen according to the results of titrations essentially different from the technique of their complement-fixation test and indeed, may use an antigen in an arbitrary amount with inadequate or no preliminary titration at all; these practices cannot yield *uniformly* good and satisfactory results.

Analysis of Methods for Determining the Amount of Antigen to Employ.—A variety of methods are being used to determine the proper amount of antigen to employ in Wassermann and other complement-fixation tests, which may be classified somewhat as follows:

First Method.—To determine the anticomplementary unit and use one-half or less this amount as the dose of antigen for the main tests. Wassermann advocated the use of one-half the largest amount of antigen which itself was found to be not at all anticomplementary; subsequent workers employ one-half to one-sixth of the anticomplementary unit.

With cholesterolized antigens most workers use from one-quarter to one-sixth the anticomplementary units to avoid non-specific reactions; even a wider margin as one-tenth or more is advisable.

Many excellent and careful workers have found this simple method for determining the dose of antigen satisfactory; however, it would appear defective by reason of the fact that the substances in an antigen responsible for the anticomplementary effects may not be antigenic. An extract used in a fraction of its anticomplementary unit may not be sufficiently antigenic and this is particularly true

of plain alcoholic extracts of syphilitic liver. Furthermore, it would appear a good principle *to avoid the use of an unnecessarily large amount of antigen* as may be represented by a cholesterolized extract in one-third of its anticomplementary unit; this would lessen the danger of non-specific reactions and indeed, under certain conditions, a large amount may not be as antigenic as smaller amounts as shown by L'Esperance and Coca[8] and Ottenberg.[9]

Second Method.—With the above method a fixed amount of complement is employed in the titration with varying amounts of antigen; an entirely different principle is used by those who titrate the complement in the presence of the antigen, as Thomsen, Boas, Thomas and Ivy and others. These workers use a more or less fixed and arbitrary amount of antigen and adjust the complement to this amount; in other words, the adjustment is not of antigen to complement, but of complement to antigen.

As previously stated I have adopted the principle of titrating the complement in the presence of the dose of antigen, but not of a fixed and arbitrary amount because this fixed amount may actually prove too much or too little for the best work.

Third Method.—To determine the antigenic and anticomplementary units and use 2 or more antigenic units as the dose of antigen provided this amount is not more than one-third the anticomplementary and hemolytic units. This method is more accurate as it tends to avoid the use of excessive amounts of antigen and of worthless antigens. It is open to the objection of being based upon titration with a certain serum which may yield a unit unsuitable for other sera; however, by titrating with a mixture of antibody sera and using 2 or more antigenic units as the dose for the main tests, this objection is readily overcome.

Antigenic Sensitiveness in Relation to Anticomplementary Activity.—A principle established by Wassermann and followed by serologists generally, is to employ the largest amount of antigen in order to secure the most sensitive specific reactions; the unsafe amount is the anticomplementary and hemolytic units and the safe amount, the largest fraction of these which avoids non-specific reactions. The question arises, do these large amounts of antigen as one-half to one-quarter the anticomplementary amounts represent the optimum amount for complement-fixation tests?

With the principle of Wassermann's technique employing a fixed and arbitrary amount of complement, the answer would appear to be in the affirmative. In all of my experiments using one-half, one-third, one-fourth, one-fifth, one-sixth, and higher fractions of the anticomplementary units of various antigens with *a fixed amount of complement in the antigen titrations and main tests*, the strongest reactions were observed with the larger amounts of antigen, none of the antigens being hemolytic in the doses employed.

However, the method of arbitrarily adopting a fraction of the anticomplementary unit of an antigen as a dose for *syphilis* complement-fixation tests cannot be recommended for at least two reasons:

1. Some antigens and particularly plain alcoholic extracts of syphilitic liver and beef or human heart muscle, may not be antigenic even in doses equal to one-half the anticomplementary units. This is particularly true if tests are conducted with a primary incubation of one hour in a water-bath with an alcoholic extract of syphilitic liver. It is not true, however, with a refrigerator incubation which increases the degree of complement-fixation; neither is it true of cholesterolized extracts with either form of primary incubation.

2 The method may result in the use of a needlessly large amount of antigen and with cholesterolized extracts greatly increases the chances of non-specific reactions. Of course this varies according to the kind of antigen and to the amount of syphilis antibody in a serum and for these reasons most serologists use one-third to one-sixth of the anticomplementary units in order to "pick up" weakly positive sera.

Since there is not necessarily a relation between the anticomplementary and antigenic activities of any antigen, a much better practice is to titrate for antigenic activity and use a sufficient number of antigenic units as a dose for complement-fixation tests.

With *bacterial* antigens however, the anticomplementary titration is of much greater importance than the antigenic titration. Not infrequently an antibody serum is not available for antigenic titrations but the antigen may be employed in one-half, one-third or one-fourth of the anticomplementary unit, the success of the complement-fixation tests depending upon the chance that these amounts are antigenic and sufficient. Practice and experience have shown that this custom is usually satisfactory, providing the antigen is made by a good technique and a positive control is included in the complement-fixation tests.

Prezone Reactions.—When antigens are titrated and the complement-fixation tests conducted with a fixed and arbitrary amount of complement as in the original Wassermann test, strongest reactions with syphilitic sera usually occur with the largest amounts of antigen. There may be no difference in the degree of fixation in tests employing one-half to one-quarter the anticomplementary units of antigen, but these amounts usually give stronger reactions than tests using one-sixth to one-tenth or smaller amounts of antigen.

When however, the complement is titrated in the presence of antigen and 2 units used in complement-fixation tests as in my technique, different results may be observed with some antigens.

L'Esperance and Coca and Ottenberg have shown that under these conditions a large dose of antigen (as one-half the anticomplementary unit) may give less fixation than a smaller dose (as one-tenth the anticomplementary unit). This is well shown in the following results of antigenic titration of my cholesterolized and lecithinized alcoholic extract of beef heart, in which a mixture of strongly positive syphilitic sera were used in a dose of 0.05 cc. (0.5 cc. of 1 to 10):

0.5 cc.	1 to 10	= +
0.5 cc.	1 to 20	= ++
0.5 cc.	1 to 40	= +++
0.5 cc.	1 to 80	= ++++
0.5 cc.	1 to 160	= ++++
0.5 cc.	1 to 320	= ++++
0.5 cc.	1 to 640	= ++++
0.5 cc.	1 to 1280	= ++++
0.5 cc.	1 to 2560	= +++
0.5 cc.	1 to 5120	= +

Similar results may be observed when Wassermann antigens are employed in fractions of their anticomplementary units. For example the following results occurred with my cholesterolized and lecithinized alcoholic extract of beef heart titrated with 0.05 cc. of strongly positive syphilitic serum:

Fractions or multiples of anticomplementary unit										
$\frac{1}{4}$	$\frac{1}{3}$	$\frac{1}{2}$	1	2	4	6	8	10	12	15	20
Reactions											
+	+	++	+++	++++	++++	++++	++++	++++	++++	+++	+

The same has been observed in complement-fixation tests employing an antisheep serum with an antigen of sheep serum:

Antigen dilutions.	$\frac{1}{100}$	$\frac{1}{200}$	$\frac{1}{300}$	$\frac{1}{400}$	$\frac{1}{500}$	$\frac{1}{600}$	$\frac{1}{800}$	$\frac{1}{1000}$	$\frac{1}{2000}$	$\frac{1}{3000}$
Antisheep serum (0.005 cc.)	−	+	++	++++	++++	++++	++++	+++ +	++ + −	+ − +
Antisheep serum (0.001 cc.)	−	−	−	−	−	+	++	+++	++++	+++
Antisheep serum (0.0005 cc.)	−	−	−	−	−	−	−	+	+	−

With saline or watery antigens of bacteria however, prezone reactions are rarely observed; almost invariably the larger amounts have yielded the strongest complement-fixation reactions.

Another type of prezone reaction is sometimes observed when antigen is used in a fixed dose with varying amounts of patient's serum. Sometimes the larger amounts of serum yield weaker reactions than the smaller amounts. In my syphilis complement-fixation method each serum is used in dose of 0.1, 0.05, 0.025, 0.005 and 0.0025 cc. amounts and about once in every 500 sera the 0.1 cc. dose will give a negative reaction when the 0.05 cc. dose reacts positively; about once in every 300 sera the 0.1 cc. dose gives a weaker reaction than the 0.05 cc. dose and both possibilities con-

stitute important reasons why every serum should be tested in at least two different amounts because of the greater practical importance of the subject.

The Number of Antigenic Units of Antigen in Relation to Complement-fixation in Syphilis.—In the technique adopted for the titration of antigen in my complement-fixation test, the antigen is titrated by using varying amounts of an emulsion with 0.05 cc. of a mixture of syphilitic sera and 2 units of complement titrated plain, that is, in the absence of antigen; with this technique prezone reactions do not usually occur with the doses of antigen employed and there is not the slightest difficulty in deciding upon the smallest amount of antigen giving a ++++ reaction, which is taken as the antigenic unit.

With this technique the question arises how many antigenic units should be used as an optimum dose of antigen for the most sensitive specific reactions. Only experience based upon numerous experiments can give the answer; furthermore, all experiments must be conducted with a uniform technique.

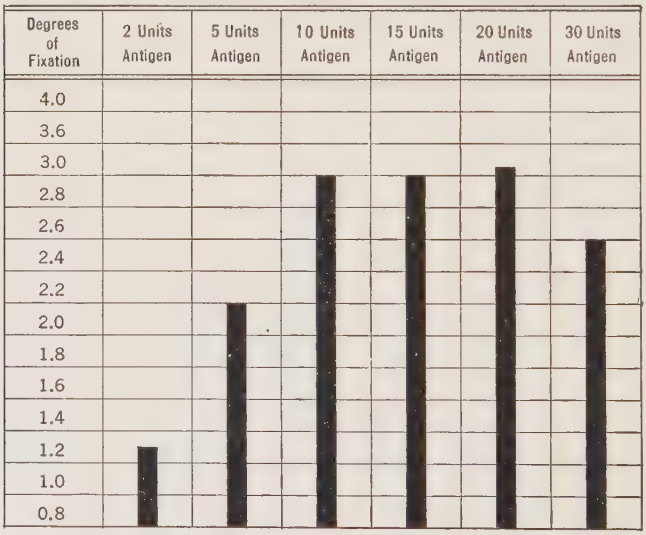

FIG. 10.—The relation of antigenic units of cholesterolized and lecithinized alcoholic extract of beef heart to degree of complement-fixation.

The general results of my experiments[10] have shown *that the optimum amount of antigen is from 5 to 15 antigenic units* depending *upon the kind of antigen*. With my new extract of cholesterolized and lecithinized alcoholic extract of beef heart the optimum dose has been usually 10 to 20 units as shown in Fig. 10 and I have adopted 10 units as the uniform dose. *Experience has shown that this dose of 10 antigenic units yields very sensitive and specific reac-*

tions in my syphilis complement-fixation test and is the amount of antigen proposed as an optimum dose. This dose must be, however, at least ten times less than the anticomplementary and hemolytic units; otherwise the antigen should not be used. Experience has shown that the dose employed (10 units) is usually thirty to fifty times less than the anticomplementary and hemolytic units which renders it eminently safe and specific for syphilis when frambesia can be excluded.

The Optimum Amount of Antigen for Bacterial Complement-fixation Tests.—In my experience *this is the largest amount that may be safely employed to avoid non-specific reactions.* Of course, the antigen must possess antigenic sensitiveness as quantity cannot always compensate for defective quality. But as previously stated, in bacterial complement-fixation the amounts of antibody in sera are seldom large and under these conditions *the maximum allowable dose of antigen possessing antigenic sensitiveness is required to produce sensitive reactions.*

The results of some experiments on this important question are shown in Tables 28, 29, 30, 31, 32 and 33 with tubercle, typhoid, gonococcus and *B. abortus* antigens. *Invariably the larger amounts of antigen yielded the stronger reactions whether these amounts were gauged as* fractions of the anticomplementary units (Tables 28, 29, 30, 32 and 33) or as multiples of the antigenic unit (Table 31).

TABLE 28.—DOSE OF ANTIGEN IN RELATION TO THE TUBERCULOSIS COMPLEMENT-FIXATION TEST.*

Immune calf serum.	Amount of antigen.						
	½ unit.†	⅓ unit.	¼ unit.	⅕ unit.	⅙ unit.	⅛ unit.	1/10 unit.
0.05 cc.	++++	+++	++	+	+	+	−
0.025 cc.	++++	+++	++	+	+	−	−
0.0125 cc.	++++	+++	++	+	+	−	−
0.00625 cc.	+++	++	+	−	−	−	−
0.003 cc.	++	+	−	−	−	−	−

* Refrigerator primary incubation. † One-half anticomplementary unit, etc.

TABLE 29.—DOSE OF ANTIGEN IN RELATION TO THE TYPHOID COMPLEMENT-FIXATION TEST.*

Immune calf serum.	Amount of antigen.						
	½ unit.†	⅓ unit.	¼ unit.	⅕ unit.	⅙ unit.	⅛ unit.	1/10 unit.
0.005 cc.	++++	++++	++++	++++	++++	++++	++++
0.0025 cc.	++++	++++	++++	++++	++++	+++	+
0.00125 cc.	++++	++++	++	++	+	−	−
0.0006 cc.	+++	++	−	−	−	−	−
0.0003 cc.	+	−	−	−	−	−	−

* Refrigerator primary incubation. † One-half anticomplementary unit, etc.

TABLE 30.—DOSE OF ANTIGEN IN RELATION TO THE GONOCOCCUS COMPLEMENT-FIXATION TEST.*

Immune goat serum.	Amount of antigen.				
	½ unit.†	⅓ unit.	¼ unit.	⅛ unit.	⅙ unit.
0.05 cc.	++++	+++	+++	+	−
0.025 cc.	++++	++	−	−	−
0.0125 cc.	++	+	−	−	−
0.006 cc.	+	−	−	−	−

* Refrigerator primary incubation.
† One-half anticomplementary unit, etc.

TABLE 31.—DOSE OF ANTIGEN IN RELATION TO THE GONOCOCCUS COMPLEMENT-FIXATION TEST.*

Immune. goat serum.	Amount of antigen.				
	2 units.†	4 units.	6 units.	8 units.	10 units.
0.05 cc.	++++	++++	++++	++++	++++
0.025 cc.	++	++	+++	++++	++++
0.0125 cc.	−	+	++	+++	++++
0.006 cc.	−	−	−	+	++

* Refrigerator primary incubation.
† Two antigenic units on basis of titration with 0.05 cc. goat immune serum, etc.

TABLE 32.—DOSE OF ANTIGEN IN RELATION TO THE CONTAGIOUS ABORTION (B. ABORTUS, BANG) COMPLEMENT-FIXATION TEST.*

Cow serum.	Amount of antigen.						
	½ unit.†	⅓ unit.	¼ unit.	⅛ unit.	⅛ unit.	1½ unit.	1⅙ unit.
0.01 cc.	++++	++++	++++	++++	+++	+	−
0.005 cc.	++++	++++	++++	++++	+++	−	−
0.0025 cc.	++++	++++	++++	+++	−	−	−
0.00125 cc.	++++	++++	+++	++	−	−	−

* Refrigerator primary incubation.
† One-half anticomplementary unit, etc.

TABLE 33.—DOSE OF ANTIGEN IN RELATION TO THE CONTAGIOUS ABORTION (B. ABORTUS, BANG) COMPLEMENT-FIXATION TEST.*

Cow serum.	Amount of antigen.						
	½ unit.†	⅓ unit.	¼ unit.	⅛ unit.	⅛ unit.	1½ unit.	1⅙ unit.
0.01 cc.	++++	++++	+++	+	−	—	−
0.005 cc.	++++	+++	+	−	−	—	−
0.0025 cc.	+++	++	+	−	−	−	−
0.00125 cc.	++	−	−	−	−	−	−

* Primary incubation one hour in a water-bath at 38° C.
† One-half anticomplementary unit, etc.

As shown in these tables the larger the amounts of immune sera employed in these titrations, the stronger were the reactions with small amounts of antigen but *when the amount of antibody was small as in the higher dilutions of immune sera, the degree of complement-fixation was in direct proportion to the amount of antigen employed.* In other words when the amount of antibody in the serum is small as it usually is in human tuberculosis and gonococcus infections, the sensitiveness of complement-fixation tests is increased by using the largest allowable amount of antigen.

Rules Regarding Dosage of Bacterial Antigens.—A large series of experiments have resulted in our adoption of the following rules governing the amount of antigen to employ in bacterial complement-fixation tests:

1. If the anticomplementary unit is determined by titration with a primary incubation of antigen and complement for eighteen hours at 6° to 8° C. the dose may be one-third this unit for tests employing a similar primary incubation as is the case with my method. The antigen must of course, be known to possess antigenic sensitiveness on the basis of actual tests or at least be prepared according to a method likely to result in antigenic sensitiveness.

2. If the anticomplementary titration is determined by titration in a water-bath for one hour but the main complement-fixation tests are conducted with a primary incubation of eighteen hours at 6° to 8° C., the dose may be one-fourth the anticomplementary unit. This is necessary in order to allow for greater complement destruction and greater anticomplementary activity of antigen at 6° to 8° C. for eighteen hours than occurs at 38° C. for one hour.

3. If the primary incubation of the anticomplementary titration and the complement-fixation tests are conducted in a water-bath for one hour, the dose of antigen may be one-half the largest amount that is not anticomplementary. This was established by Wassermann for watery extracts of syphilitic liver for the syphilis complement-fixation test; it is to be noted that this amount of antigen is not quite as much as one-half the anticomplementary unit as defined above.

I believe these amounts of antigen represent the maximum allowable in routine work to avoid non-specific reactions; it is scarcely necessary to state that *they must be antigenic.* Sometimes an antigen in one-third or one-fourth the anticomplementary unit carries as much as 20 antigenic units; on the other hand most antigens carry much less and some none at all. The more antigenic units there are in one-fourth the anticomplementary amounts of an antigen, the better the antigen and stronger the reactions; since prezone reactions do not occur or, at least, are relatively uncommon, I believe *in using the maximum amount of antigen as defined above regardless of how many antigenic units this represents.*

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CHAPTER XVIII.

METHODS FOR ESTABLISHING UNIFORM AND STANDARDIZED UNITS OF ANTIGENS FOR COMPLEMENT-FIXATION TESTS.

If an agreement could be reached upon the *kind* of antigen to use in the complement-fixation test for syphilis and upon a *uniform method for titration*, a great advance would be made toward standardization of technique because, with these two essential matters settled, my experience has demonstrated that serologists working in different laboratories obtain remarkably uniform results by making and titrating their own antigen according to any given method.

A greater refinement in technique and results would be observed if a *central laboratory* titrated the antigen in addition to the titrations made by individual serologists just as the Hygienic Laboratory titrates diphtheria and tetanus antitoxins and furnishes established units of toxin or antitoxin to the manufacturers.

It was with this idea in mind that I have experimented during the past eight years for the purpose of developing good methods for establishing a standardized unit of antigen for the Wassermann test should serologists ever reach an agreement upon the kind and amount of antigen to employ and a uniform complement-fixation technique.

METHODS FOR ESTABLISHING STANDARD UNITS OF ANTIGENS.

Kind of Antigen in Relation to Standardizing the Unit for Syphilis.—A question of primary importance arises as follows: If the technique for titrating antigen and conducting complement-fixation tests could be agreed upon, would different kinds of antigen yield approximately the same or different results? For example, if the antigenic units of two widely different extracts as a plain and a cholesterolized alcoholic extract of heart were determined and 10 units of each used in complement-fixation tests with the same sera, would the reactions be the same?

My answers to these questions based upon numerous experiments are as follows: In a qualitative test, that is, using one dose of serum (0.1 cc.) with a primary incubation of fifteen to eighteen hours at 6° to 10° C., the results have been quite uniform insofar as positive

or negative reactions were concerned. With warm incubation of one hour at 38° C. considerable differences have occurred, the cholesterolized extracts yielding more positive reactions. Refrigerator primary incubation greatly reduces the differences observed in complement-fixation tests employing plain and cholesterolized extracts with the same sera.

In a quantitative test employing graded amounts of serum, differences in the reactions were observed according to the antigen employed, even with a primary incubation in the refrigerator; these differences were considerably more marked with warm incubation for one hour. Extracts containing 0.1 to 0.2 per cent cholesterol yielded stronger reactions with the smaller doses of serum although with the 0.1 and 0.05 cc. amounts of serum the reactions with plain and cholesterolized extracts were more nearly uniform.

According to the results of these experiments the same kind of antigen (cholesterolized and lecithinized alcoholic extract of beef heart) prepared at different times but titrated in a uniform manner, used in the same uniform dose of 10 antigenic units and tested with syphilitic sera in the same technique by refrigerator primary incubation, have yielded qualitative reactions of remarkably similar degree (that is, positive or negative) but may show slight differences in quantitative tests employing graded amounts of serum or spinal fluid.

Methods for Standardization.—If the kind of antigen, method of titration and amount to use in complement-fixation tests could be agreed upon, a unit for standardizing antigen may be established by any one of the following procedures:

1. By using positive serum as a standard and requiring an antigen to possess such antigenic sensitiveness as to fix complement with a certain amount of this standard serum. The problem then would be how to choose and keep this serum, what amount to use, how to replenish it from time to time to keep a uniform and standard unit and how it may be used in the titrations.

2. By using an antigen as a standard and requiring new antigens to possess an acceptable degree of antigenic sensitiveness and certain freedom from anticomplementary and hemolytic activities. Under these conditions it would be necessary to determine what antigen to use, how it should be kept, how it may be replenished to maintain the standard unit and how it may be used in a series of titrations when testing new antigens.

In a comparative study of these methods five years ago I thought that it was better to use antigen as a standard than serum[1] and I chose acetone-insoluble lipoids as the standard antigen, described a method for keeping and replenishing it from time to time as likewise a method for using it in establishing and maintaining a standard unit. But during the past two years I have used *dried serum* with

better results. In relation to the syphilis complement-fixation test frozen spinal fluid may be used because less likely to become anticomplementary but it is much more difficult to secure, and *serum rapidly dried* as described in Chapter XX has proven the best method. Sera preserved with glycerol, phenol, tricresol, etc., have usually tended to become anticomplementary; frozen serum and spinal fluid have ranked second in efficiency to dried serum but are much more troublesome to keep. Syphilitic and other immune sera, however, may be dried by the method employed without becoming anticomplementary and with practically no loss of complement-fixing power at the time or subsequently over a period of eight months to a year or more providing they are hermetically sealed and kept in a refrigerator.

METHOD FOR MAINTAINING AND USING STANDARD DRIED SERA FOR THE TITRATION OF ANTIGENS IN THE WASSERMANN TEST.

A mixture of at least 6 to 10 different syphilitic sera yielding strongly positive reactions should be employed. Each serum must be fresh, free of anticomplementary effects, preferably sterile and free of natural antisheep hemolysin.

Amounts of 0.3 cc. of the mixed *unheated* sera are placed in a series of thin-walled small sterile test-tubes and rapidly dried as described in Chapter XX. After drying, the residue in each is broken up with a glass rod, the tubes sealed and kept in a refrigerator. For use the contents of a tube are dissolved in 3 cc. of saline solution; 0.5 cc. therefore carries the equivalent of 0.05 cc. of serum.

A mixture of *normal* or negative sera is prepared in the same manner to be used in the anticomplementary and hemolytic titrations of antigens.

As new antigens are prepared they are titrated as described in Chapter XXXI, *5 fixing units* of the solution of dried positive serum being used in the antigenic titrations and 0.5 cc. of the solution of dried negative serum used in the anticomplementary and hemolytic titrations. It is easily possible and convenient for a central laboratory to supply both. As described in Chapter XX one may readily desiccate large measured amounts of serum. After drying, the material is weighed and amounts equivalent to 0.3 cc. of serum dispensed in ampoules.

Every six months, or as the supply of desiccated standard positive serum is exhausted, a fresh lot may be prepared and its unit of fixing power or antibody content adjusted to the preceding serum as follows:

1. The contents of a tube of dried standard serum and one of the new serum, each containing the equivalent of 0.3 cc., are dissolved

in 3 cc. of saline solution, *heated* at 55° C. for fifteen minutes and titrated for their fixability by placing the following amounts in two series of fifteen test tubes:

Tube No. 1	0.5 cc. as is	= 0.05 cc. serum
Tube No. 2	0.5 cc. 1:2	= 0.025 cc. serum
Tube No. 3	0.5 cc. 1:3	= 0.016 cc. serum
Tube No. 4	0.5 cc. 1:4	= 0.0125 cc. serum
Tube No. 5	0.5 cc. 1:5	= 0.01 cc. serum
Tube No. 6	0.5 cc. 1:6	= 0.008 cc. serum
Tube No. 7	0.5 cc. 1:7	= 0.007 cc. serum
Tube No. 8	0.5 cc. 1:8	= 0.006 cc. serum
Tube No. 9	0.5 cc. 1:9	= 0.0055 cc. serum
Tube No. 10	0.5 cc. 1:10	= 0.005 cc. serum
Tube No. 11	0.5 cc. 1:11	= 0.0045 cc. serum
Tube No. 12	0.5 cc. 1:12	= 0.004 cc. serum
Tube No. 13	0.5 cc. 1:13	= 0.0038 cc. serum
Tube No. 14	0.5 cc. 1:14	= 0.0036 cc. serum
Tube No. 15	0.5 cc. 1:16	= 0.003 cc. serum

2. Ten antigenic units of antigen are now added to each tube along with 2 full units of complement (mixture of at least 6 guinea-pig sera for uniform serum) and sufficient saline solution. A primary incubation is given of eighteen hours at 6° to 10° C. followed by ten to fifteen minutes in a water-bath, the addition of 2 units of anti-sheep hemolysin, 0.5 cc. of 2 per cent sheep cells and a secondary incubation of one hour in a water-bath exactly as described in Chapter XXXI. A control on the serum alone for anticomplementary action should be included using 0.5 cc. of the solution; it should show complete hemolysis. Hemolytic system and corpuscle controls should be also included.

The smallest amount of each serum giving a ++++ reaction is recorded as the M. F. U. or *minimal fixing unit.*

3. If the standard serum shows the *same fixing unit as formerly* the fixing unit of the new serum is easily adjusted according to the following example:

Original fixing unit of standard dried serum = 0.005 cc. (0.5 cc. of 1 to 10 or tube No. 10).
Present fixing unit of standard dried serum = same.
Fixing unit of new dried serum = 0.0055 cc. (0.5 cc. of 1 to 9 or tube No. 9).
For titrating antigens use *5 units* = 0.0275 cc. or 0.5 cc. of 1 to 2 solution of new dried serum (equivalent to 0.025 cc. of wet serum).

4. If, however, the fixing unit of the first or standard serum is now *more* than it was originally because of a difference in the fixability of the complement serum or a difference in antigen, the fixing unit of the second serum is adjusted to the original standard unit as per this example:

Original fixing unit of standard serum = 0.005 cc. (0.5 cc. of 1 to 10 or tube No. 10).
Present fixing unit of standard serum = 0.008 cc. (0.5 cc. of 1 to 6 or tube No. 6).
Fixing unit of new serum = 0.0055 cc. (0.5 cc. of 1 to 9 or tube No. 9).

$$\frac{0.005}{0.008} = \frac{5}{8} \text{ of } 0.0055 = 0.0034 \text{ cc.}$$

(the fixing unit of the new or second serum equivalent to 0.5 cc. of 1 to 15 dilution of the stock solution of dried serum.

For titrating antigens 5 M. F. U.'s would be used equivalent to 0.017 cc. serum or 0.5 cc. of 1 to 3 dilution of the solution of dried serum obtained by dissolving the contents of a tube of dried product (containing the equivalent of 0.3 cc. serum) in 3 cc. of saline solution.

5. If, however, the fixing unit of the standard serum is now *less* than originally because of a more sensitive antigen or complement the calculation is made as follows:

Original fixing unit of standard serum = 0.005 cc. (0.5 cc. of 1 to 10 or tube No. 10).
Present fixing unit of standard serum = 0.004 cc. (0.5 cc. of 1 to 12 or tube No. 12).
Fixing unit of new = 0.0055 cc. (0.5 cc. of 1 to 9 or tube No. 9).

$$\frac{0.005}{0.004} = \frac{5}{4} \text{ of } 0.0055 = 0.007 \text{ cc.}$$

(the fixing unit of the new serum equivalent to 0.5 cc. of 1 to 7 dilution of the stock solution of dried serum).

For titrating antigens 5 M. F. U's. would be used equivalent to 0.035 cc. serum or 0.35 cc. of a solution of dried serum obtained by dissolving the contents of a tube of dried product in 3 cc. of saline solution.

6. The new or second serum now becomes the standard for titrating antigens and at the end of six months a third serum is adjusted to it in the same manner as described, the system being kept in continuous operation. In conducting the anticomplementary and hemolytic titrations of new antigens 0.05 cc. (0.5 cc. of 1 to 10) fresh, heated *normal* serum or 0.5 cc. of a solution of dried normal serum may be used (dried residue of 0.3 cc. dissolved in 3 cc. of saline and heated at 55° C. for fifteen minutes).

METHOD OF USING STANDARD DRIED SERUM FOR THE TITRATION OF BACTERIAL ANTIGENS.

The same method may be employed for the titration of bacterial antigens and especially those of glanders and contagious abortion

of cows and mares so widely employed in livestock laboratories. The respective immune sera are selected, dried and employed in exactly the same manner.

It is hardly worth while at present, however, to attempt establishing standard methods for tuberculosis, typhoid, gonococcus and other bacterial antigens because of their limited use, but exactly the same method may be employed. As described in Chapter XX these sera are easily dried and preserved for this purpose or for use as positive controls in conducting complement-fixation tests. Furthermore, methods are described therein for using dried immune sera obtained by the immunization of the lower animals since it is ordinarily quite difficult to secure and maintain a supply of tuberculosis, gonococcus, typhoid and other sera from human sources.

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CHAPTER XIX.

HEATING OF SERUM AND THE SERUM CONTROL IN COMPLEMENT-FIXATION TESTS.

THE INFLUENCE OF HEATING SERUM UPON COMPLEMENT-FIXATION REACTIONS.

While in the original Wassermann test each serum is "inactivated" by heating at 56° C. for thirty minutes, numerous modifications have been described in which unheated serum is used for the purpose of securing more sensitive reactions or for economy and greater simplicity in technique. Originally Wassermann and his associates advised the heating of sera for the main purpose of destroying the native complements and hence the phrase "inactivation of serum" has come into general use, but at the present time it is known that sera are heated for additional and probably more important reasons.

The subject is one of primary importance in relation to complement-fixation tests by reason of having a direct bearing upon the sensitiveness and specificity of the reactions; questions of economy of materials and time and of simplicity in technique are certainly of lesser importance.

Classification of Tests Employing Unheated or Raw Serum.—These may be divided into three main classes as follows:

1. Tests of the nature of those of the original Noguchi test employing unheated serum, ignoring the presence of native complement and using guinea-pig complement.
2. Tests as those of Tschernogubow, Emery, Thompson, Ronchese, Noguchi homohemolytic, Bronfenbrenner and Schlesinger and Stern in which the native complements in the unheated serum of each patient are utilized.
3. Tests as those of Tschernogubow, Foix, Hecht, Flemming, Gradwohl, Bruce, Seelman, Bartlett and O'Shansky in which the native complements and certain natural hemolysins (antiguinea-pig and antisheep) in the unheated serum of each patient are utilized.

Advantages and Disadvantages of Tests Employing Unheated or Raw Serum.—The *main advantage* is greater sensitiveness due to the fact that none of the antibody concerned in complement-fixation is lost or destroyed; furthermore, in those tests employing the native complement of each serum the reactions may be stronger because human complement is somewhat more fixable than guinea-pig complement and at the same time more susceptible to the anticom-

plementary influence of serum and antigen alone, which tends to increase the degree of complement absorption or fixation.

The *main disadvantage* is the fact attested to by Seligman and Pinkus,[1] Noguchi,[2] Browning and McKenzie[3] and numerous other investigators, that unheated or raw serum may yield falsely positive or non-specific reactions; there appears to be conclusive evidence that tests conducted with the raw sera of healthy individuals or of persons suffering with diseases other than syphilis, yaws and leprosy are open to this serious objection. Noguchi has designated these reactions as "proteotropic" on the basis of his belief that they are due to the absorption or fixation of complement by something in unheated non-syphilitic serum and a lipoid-protein substance in the extract used as antigen; for this reason he advises the use of protein-free antigens, as his extract of acetone-insoluble lipoids. Whether or not this is the true explanation it would appear as conclusively proved that unheated serum may yield these non-specific reactions, whereas heated sera do not.

Other advantages of using raw serum in some tests are economy, inasmuch as guinea-pig complement is unnecessary and the tests usually simpler and less time-consuming; other disadvantages are the variation in fixability of human complement, the necessity for using fresh sera, the 2 to 10 per cent of human sera deficient in complement, natural hemolysins or both, and the impossibility of testing cerebrospinal fluids. The disadvantages therefore, outweigh and outnumber the advantages and for this reason I have reached the conclusion that human complement should not be utilized in the Wassermann or other complement-fixation test.

THE INFLUENCE OF HEAT UPON COMPLEMENT.

The hemolytic complements of human and guinea-pig sera are very susceptible to heat and readily inactivated; most interest centers in the complements of human sera in relation to the amount of heat required for inactivation in preparation for complement-fixation tests.

As shown by Rule, Trist and myself[4] when fresh human sera are heated in a water-bath at 56° C. the complement activity is reduced almost eight times in five minutes; inactivation begins even at 40° C. and becomes progressively greater as the temperature approaches 55° C. but most destruction occurs with temperatures of over 50° C.

Guinea-pig complement is somewhat more resistant to heat; Noguchi and Bronfenbrenner[5] found that the activity of sera heated at 55° C. for thirty minutes was reduced to one-thirtieth or one-fortieth the original strength of unheated serum, but was not completely destroyed. In our experiments activity was reduced at least tenfold when sera were heated at 55° C. for ten minutes; at 56° C.

inactivation began after an exposure of one minute, being one-third reduced in five minutes from which period inactivation was very rapid.

Insofar as destruction of hemolytic complement in fresh undiluted human sera is concerned to a sufficient degree for complement-fixation tests, it would appear that heating the sera at 55° C. in a water-bath for five to ten minutes is sufficient; 50° C. is sufficient. Simon[6] has advocated heating human sera at 55° C. for ten minutes for the purpose of inactivation and I agree with him that this exposure is ample, although it may be insufficient for the removal of the antilysins; Davis[7] has likewise shown that heating at 55° C. for fifteen minutes as employed in my method is sufficient for the inactivation of human complement.

INFLUENCE OF HEAT UPON ANTICOMPLEMENTARY SUBSTANCES IN HUMAN SERA.

The development of anticomplementary substances (antilysins) in human sera are of great importance in relation to complement-fixation tests, and very probably sera are routinely heated for the Wassermann test more for the purpose of removing these than for the inactivation of complement or destruction of the substance causing proteotropic reactions.

These antilysins appear to inhibit hemolysis by a direct effect upon complement and hence are designated as anticomplementary substances; some are destroyed or rendered inactive by heating the sera at 54° to 56° C. for thirty minutes and are designated as "thermolabile;" others resist this temperature and are designated as "thermostabile."

Fortunately the great majority of sera collected with ordinary care, kept in an ordinary refrigerator and tested within five days do not become anticomplementary at all or develop antilysins readily removed by heating the sera. In this connection I may state that *sera left with the coagulum for this period of time are apt to be more satisfactory than sera removed from the clot and kept separated for several days.* The important matter, of practical importance insofar as the majority of complement-fixation tests are concerned, is to determine the minimum amount of time for heating sera at 55° C. to remove the thermolabile antilysins.

As observed by Rule, Trist and myself the antilysins of human sera are not appreciably influenced by heating the sera at 50° C. or less for periods of thirty minutes; at 55° C., however, many of the antilysins are destroyed.

Heating sera at 55° C. for five minutes may result in a marked reduction of the antilysins, the effects becoming more marked as heating is continued; after fifteen minutes of heating the thermo-

labile antilysins in the majority of sera are destroyed. Tests with a large number of sera varying in age from one to ten days kept at 6° to 8° C. and used in amounts of 0.1 cc. unheated and after heating at 55° C. for one, five, ten, fifteen, twenty and thirty minutes, have shown that *the antilysins of over 90 per cent of ordinary sera, that is, clear and containing not more than traces of hemoglobin but sometimes showing the presence of bacteria in cultures, are effectually removed by heating at 55° C. for fifteen minutes.* For ordinary work this exposure is sufficient; occasionally sera prove anticomplementary after this amount of heating, but most of these contain thermostabile antilysins which effectually resist higher temperatures and more prolonged exposures and particularly heating at 54° to 56° C. for thirty minutes, as is the usual custom.

Cerebrospinal fluid seldom develops antilysins unless contaminated with bacteria; unlike sterile serum thermolabile antilysins are only exceptionally encountered and in our experience only in fluids containing relatively large amount of protein, as that from paretics. When bacteria are permitted to grow in a fluid both thermolabile and thermostabile antilysins may develop but under *ordinary circumstances cerebrospinal fluid may be used unheated; when heating is required 55° C. for fifteen minutes is usually sufficient for the removal of thermolabile antilysins.*

THE INFLUENCE OF HEAT UPON PROTEOTROPIC COMPLEMENT-FIXATION.

In this part of the investigation by Rule, Trist and myself only the sera of persons known to be healthy and non-syphilitic were employed; for these reasons our supply was limited to a circle of physicians and laboratory assistants, as the statements of dispensary and hospital patients were not considered sufficiently safe and certain. A small percentage of these sera yielded weakly positive complement-fixation reactions with a variety of antigens including cholesterolized alcoholic extracts of heart muscle, plain alcoholic extracts of heart muscle, alcoholic extracts of syphilitic liver and acetone-insoluble lipoids of beef heart.

In our experience a serum found to yield this reaction with any one antigen was apt to do so with all although the reactions were usually stronger with the cholesterolized extracts.

The destruction of the non-specific substance in unheated serum responsible for the proteotropic reaction begins when serum is heated at 45° to 50° C. for thirty minutes and is usually completely destroyed when serum is heated at 50° C. for this period of time; *at 55° C. destruction begins after serum is heated for one minute and is usually complete after five minutes.*

Many of those serologists employing unheated serum in modifica-

tions of the Wassermann test make no mention of the occurrence of these pseudopositive or proteotropic reactions; I have found that they may occur with the Hecht-Gradwohl test but only in a small percentage of cases, 4 per cent being found when crude alcoholic extracts were employed for antigens[8] and a much smaller percentage or none at all with extracts of acetone-insoluble lipoids.[9] Since Seligman and Pinkus showed that unheated human serum may inhibit the activity of guinea-pig complement, which property is abolished by heating human serum for thirty minutes, a large literature on the Noguchi test has shown that many investigators found these proteotropic reactions with unheated serum, and in the army laboratories where a modified Noguchi test is employed by Vedder and Craig, and in naval laboratories by Stitt and Clark, all sera are heated at 54° to 56° C. for thirty minutes. Thomsen and Boas have also called attention to the fact that falsely positive reactions may occur with unheated serum from non-syphilitic patients with cancer, nephritis, scarlet fever, leprosy and tuberculosis but with heated sera positive reactions occur only with syphilitic sera. According to these authors heating sifts out all but the true syphilitic cases, syphilitic serum being the only thermo-resisting one. This latter statement, however, cannot be accepted inasmuch as abundant evidence indicates that the heated serum in yaws may also yield positive Wassermann reactions. Graetz[10] denies the occurrence of these pseudopositive reactions with unheated sera and believes that with proper technique it is possible to obtain reliable results.

An abundant evidence tends to greatly minimize the importance of proteotropic reactions with unheated serum provided good extracts of acetone-insoluble lipoids are employed for antigens, but in my experience the possibility still exists and is always a disturbing factor in the interpretation of the results of tests with the serum of a person who denies syphilitic infection, shows no clinical evidences of the disease and yields a positive reaction with unheated serum and a negative reaction with heated serum. *For this reason I believe advisable the heating of sera for the complement-fixation test making up for the inevitable loss in sensitiveness due to some destruction of antibody by the use of sensitive antigens and by other technical procedures; for the purpose of removing the substances in human serum responsible for the pseudopositive or proteotropic reaction heating in a water-bath for five minutes at 55° C. is sufficient.*

As far as I can ascertain no one has found unheated cerebrospinal fluid yielding pseudopositive or proteotropic reactions and I have never encountered such fluid from non-syphilitic persons even when tested in large amounts, as much as 2 cc. in terms of the original Wassermann test. As previously mentioned, these fluids do not readily become anticomplementary and consequently do not require heating.

INFLUENCE OF HEAT UPON NON-SPECIFIC COMPLEMENT-FIXATION REACTIONS BY NORMAL RABBIT, DOG AND MULE SERA.

As discussed more fully in Chapter XXVIII the sera of normal rabbits, dogs and mules are especially likely to yield non-specific complement-fixation reactions with many different antigens including those employed in the Wassermann as well as in bacterial tests. Heating at 55° C. tends to increase this property while at 60° to 62° C. a decrease occurs. The nature and mechanism of these reactions have never been satisfactorily elucidated and they do not appear to be caused by antibodies, but the tendency of heating at 55° C. to increase their occurrence probably by unmasking the substances responsible by inactivation of native complement, is worthy of brief mention at this time.

THE INFLUENCE OF HEAT UPON COMPLEMENT-FIXING ANTIBODY.

The majority of those serologists employing unheated serum in various modifications of the Wassermann reactions have stated that syphilis antibody is partially destroyed when sera are heated at 54° to 56° C. for thirty minutes, but few have published the results of experiments showing the amount of destruction.

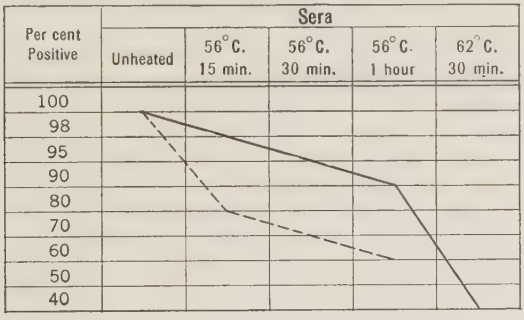
Noguchi has shown that the antibody may be completely destroyed by heating serum to 72° to 80° C. for twenty minutes; at 50° C. the antibody was reduced about one-half and at 55° C. about three-quarters, in syphilitic sera heated for twenty minutes. When sera were heated at 55° C. the antibody was reduced one-third in five minutes and at the end of an hour only one-tenth of the original amount of antibody remained. Sachs[11] found that heating syphilitic sera at 62° C. for thirty minutes destroyed the complement-fixing antibody and Marie and Levaditi[12] have reported that the antibody in cerebrospinal fluid was destroyed by heating at 75° to 80° C. for twenty minutes.

In the experiments of Rule, Trist and myself fresh syphilitic sera were used in decreasing amounts in order to render the tests quantitative for the detection of slight destruction of antibody; antisheep and antihuman hemolytic systems were employed and in each experiment the same complement and antigen were used in order to render the results strictly comparative.

Our results have shown that the destruction of syphilis antibody *may* begin when sera are heated in a water-bath at 40° C. for thirty minutes and become progressive with higher temperatures. At 55° C. some destruction occurs in five minutes and slightly more after heating for fifteen minutes. A still greater loss ordinarily occurs

after heating for thirty minutes and after an exposure of this duration to 62° C. approximately 35 per cent of sera have shown a total loss of syphilis antibody. Similar results were observed with the spinal fluids of syphilitic individuals.

Fig. 11 gives a summary of Wassermann tests with 170 selected syphilitic sera. They were conducted by the original Wassermann technique except that one-half quantities and a cholesterolized extract were used. Each unheated serum reacted positively; after reacted negatively; after heating at 56° C. for one hour, 8 per cent heating at 56° C. for fifteen minutes 2 per cent sera reacted negatively; after heating at 56° C. for thirty minutes, 5 per cent reacted negatively and after 62° C. for thirty minutes, 60 per cent reacted negatively, showing the profound influence of this amount of heat upon the antibody concerned in the Wassermann reaction. The greatest drop occurred within the first fifteen minutes of heating at 56° C., about 20 per cent of sera showing weaker positive reactions than observed with unheated sera.



———— = curve showing percentage of positive reactions.
. . . . . = curve showing percentage of weaker positive reactions.

FIG. 11.—Influence of heat upon syphilis antibody; Wassermann tests with 170 syphilitic sera, each serum being tested in 0.1 cc.

Syphilis complement-fixing antibody therefore, is ordinarily susceptible to destruction by heating sera at 55° to 56° C. although the antibody in some sera is highly resistant, suggesting that it may occur in both a thermolabile and thermostabile form; similar results have been observed in a smaller number of experiments employing sera containing the gonococcus, typhoid and tuberculosis antibodies in human sera and upon glanders antibody in horse sera.

Experiments with rabbit immune sera, however, have shown the complement-fixing antibody much more resistant to heat, especially antihuman, antidog, anticat, anticattle, antichicken and other sera employed in the complement-fixation test for the detection and

differentiation of blood stains and sera. Indeed these sera heated at 62° C. for fifteen minutes have frequently shown little or no deterioration of antibody and this is a fortunate circumstance in view of the fact that heating rabbit, mule and dog sera at 60° to 62° C. reduces the possibilities of their yielding non-specific complement-fixation reactions.

But while heating syphilitic sera results in the destruction of a portion of the antibody concerned in the complement-fixation test, it is advisable to heat all sera for the following purposes: (*a*) to inactivate native complement and thereby permit a closer adjustment of the hemolytic system; (*b*) to destroy any antilysins (anticomplementary substances) that may be present and (*c*) to prevent the occurrence of pseudopositive or proteotropic reactions with the sera of non-syphilitic persons.

For these purposes heating sera at 55° C. for fifteen minutes is sufficient and preferable to the customary period of thirty minutes, inasmuch as less destruction of antibody occurs; it is therefore, the method employed in my technique and the inevitable reduction in sensitiveness is compensated for by certain technical procedures and particularly by the kind and amount of antigen employed, a close adjustment of the hemolytic system and by the use of a primary incubation of eighteen hours at 6° to 8° C. followed by ten minutes in a water-bath.

THE SERUM CONTROL TUBE.

The object of the serum control tube is to show whether or not the serum or spinal fluid is anticomplementary and to guard thereby against false or pseudopositive reactions, due to non-specific fixation or destruction of complement by serum or spinal fluid.

Wassermann advised the use of double the amount of patient's serum and spinal fluid, but experience has shown that this caution against anticomplementary effects of serum is frequently defeated by reason of the influence of natural antisheep hemolysin. At the present time the majority of workers employing the antisheep hemolytic system use the same amount of serum in the control as in the main tube or tubes of the test.

It is easily possible for a larger amount of serum in the control tube to carry sufficient natural hemolysin to overcome the anticomplementary effect of antilysins, result in complete hemolysis and thereby lead to misinterpretation of slight inhibition of hemolysis in the main tubes. As stated by Ottenberg, the real object of a control tube is to give an accurate picture of the effect the serum is having in the main tube and for this reason I agree with him that the dose of serum in the control tube should be the same as the largest amount used in the main test.

Unfortunately, *a serum may contain so small an amount of anti-*

lysin as to yield a perfect control tube of complete hemolysis and yet show a very slight degree of inhibition of hemolysis in the presence of antigen; this however, can be guarded against in my experience, by certain technical details including the use of a proper amount of antigen and titrating the complement in the presence of antigen.

With the antisheep and antiox hemolytic systems the use of a larger amount of serum in the control tube tends rather to inaccuracy than accuracy; with the antihuman and antichicken hemolytic systems the use of a larger amount of serum or spinal fluid in the control tube tends to greater caution against anticomplementary reactions, but is not absolutely necessary for the sake of accuracy.

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CHAPTER XX.

PRESERVATION OF IMMUNE SERA AND OF SERA FOR POSITIVE AND NEGATIVE CONTROLS IN COMPLEMENT-FIXATION TESTS.

In conducting Wassermann, gonococcus, tuberculosis, typhoid, glanders, echinococcus and other complement-fixation reactions it is usually advisable to include known positive and negative controls. In this manner one has a check upon the antigen, complement and hemolytic system which gives greater confidence in reading and interpreting the reactions. When large numbers of Wassermann tests are being conducted weekly or several times a week these may be omitted since it is usually certain that each series will contain sera from known syphilitic and non-syphilitic individuals which may serve as controls on each day's work; but as a general rule it is advisable to include known controls and this is especially true in small laboratories running but few tests at a time. In the case of the gonococcus, tuberculosis and other bacterial complement-fixation tests, known positive and negative controls should be always included.

The problem is to preserve the positive and negative sera in such manner to prevent deterioration of antibody in the former and the acquisition of anticomplementary properties in either. The same is true in the case of antihuman and other immune sera for the complement-fixation test in the identification of blood stains, meat adulteration and other soluble protein substances.

SOURCES OF SUPPLY.

Very satisfactory methods for the preservation of sera have been worked out and especially by desiccation. With but little trouble and apparatus each laboratory can undertake to preserve its own. These methods are also applicable on a large scale and it is easily possible for large central laboratories to undertake the preparation and distribution of positive and negative sera for controls in the Wassermann, gonococcus, tuberculosis, typhoid, echinococcus, glanders and other bacterial complement-fixation tests as well as of immune sera for the medico-legal identification of blood stains, meat adulteration, etc.

COLLECTION OF SERA FOR POSITIVE AND NEGATIVE CONTROLS.

Positive and Negative Controls for the Wassermann Test.—Sera for positive controls in the Wassermann test are easily obtained and should be a *mixture* yielding moderately to strongly positive reactions; very strongly positive sera should not be employed as they may not match average conditions. It is my practice to use a mixture of 6 to 12 sera giving 443 — — to 444— — reactions. Sera for negative controls should be chosen with great care and preferably from individuals known to be free of syphilis as well as yielding negative Wassermann reactions. It is highly desirable for large central laboratories, especially those having connections with clinics for the diagnosis and treatment of syphilis, to undertake the collection, preservation and distribution of such sera although, as previously stated, it is easily possible for each laboratory to maintain its own supply.

Positive and Negative Controls for the Gonococcus Test.—It is much more difficult to obtain positively reacting gonococcus sera unless the laboratory has a connection with a genito-urinary clinic in which case sera from *chronic* infections of either sex are readily obtained yielding 42 — — — or even stronger reactions and particularly from cases of gonococcus arthritis. But if positive human serum is not available it is easily possible to use a serum prepared by the immunization of horses with *many different strains* of gonococci in the preparation of antigonococcus serum. It is true that the antibody content may be much higher than encountered in cases of gonococcus infection but this objection has been overcome by the method of dilution and preservation described on page 283. Negative sera are very easily obtained.

Positive and Negative Controls for the Tuberculosis Test.—Positive sera are usually readily obtained in hospitals, sanatoria or clinics devoted to the treatment of tuberculosis and are best secured from active cases of chronic pulmonary tuberculosis; mixture of several should be used. Otherwise it is possible to use antituberculosis sera prepared by the immunization of calves or goats with bacilli of human and bovine origin and to dilute the antibody content down to the level of average human sera giving 42 — — — or slightly stronger reactions, but human sera are to be preferred. Negative sera from human sources are easily obtained.

Positive and Negative Controls for the Glanders and Contagious Abortion Tests.—These are required in livestock laboratories and positive sera are usually readily obtained from animals suffering with these diseases; mixtures of the respective sera should be employed. Otherwise they may be prepared by the immunization of rabbits and the antibody diluted down to average levels.

Positive and Negative Controls for the Typhoid, Echinococcus and Other Tests.—Serum is sometimes obtainable from typhoid fever cases but if not, is readily prepared by immunization of the lower animals. Positive sera for whooping cough, streptococcus and other bacterial diseases are best prepared by immunization. Echinococcus disease of human beings is so seldom observed in this country that it is almost impossible to obtain a supply from human sources; serum may be obtained from sheep with the disease but is readily prepared by immunization of rabbits. Negative sera are easily obtained.

PREPARATION OF IMMUNE SERA FOR POSITIVE CONTROLS.

Positive serum for the Wassermann reaction should be always a mixture from syphilitic human beings; it may be secured from syphilitic rabbits but in my experience this source is seldom satisfactory. For the gonococcus, typhoid, tuberculosis and other bacterial complement-fixation tests positively reacting sera of human sources are also to be preferred but when not obtainable may be prepared by immunization of the lower animals.

In preparing these sera the animals should be immunized with several different strains of the organism in order to render the serum polyvalent; this is especially true of gonococcus and typhoid sera. Antituberculosis serum should be preferably prepared with human and bovine bacilli although I have employed sera prepared with human bacilli alone with success.

Immunization of Rabbits.—These animals are usually selected for the preparation of immune sera but unfortunately their sera sometimes yield non-specific reactions with many different antigens and especially those of bacteria, as discussed with detail in Chapter XXVIII. But it is possible to use them successfully if immunization is carried to the point where 0.5 cc. of 1 to 50 or higher dilution of serum gives strongly positive reactions because this amount of serum does not give non-specific reactions in my method.

Only large healthy and previously unused animals should be employed and since some may succumb before immunization is completed it is well to immunize two or more with each antigen at the same time.

Immunization of Guinea-pigs.—The sera of these animals do not yield non-specific reactions and from this standpoint are preferable to rabbits. As a general rule they react promptly and satisfactorily to intraperitoneal injections of antigens of gonococci, typhoid bacilli and other bacteria with the production of complement-fixing antibody. Of course the yield of serum is not as high as from rabbits nor is the antibody production as great, but when large (500 gms.

or more), healthy and previously unused animals are employed, goodly amounts of immune serum are easily secured.

At least six weekly intraperitoneal injections are required and the smallest amounts of serum giving strongly positive reactions in my method have been approximately as follows:

TABLE 34.

Serum.	After six injections.
Antigonococcus	0.5 cc. of 1:40
Antityphoid	0.5 cc. of 1:600
Antituberculosis	0.5 cc. of 1:320
Antistreptococcus	0.5 cc. of 1:20
Antiechinococcus	0.5 cc. of 1:60

Preparation of Antigonococcus, Antityphoid, Antituberculosis and Other Antibacterial Sera.—Antigens may be prepared by suspending the organisms in saline solution to contain approximately 1000 million per cubic centimeter, heating at 60° C. in a water-bath for one hour and preserving with 0.3 per cent phenol or tricresol.

Rabbits may receive 6 to 12 weekly intravenous injections beginning with 0.2 cc. diluted up to 2 cc. with saline solution; the second dose may be 0.4 cc.; the third 0.6 cc.; the fourth 0.8 cc. and the succeeding doses 1 cc. each. If any antigen happens to be particularly toxic the injections may be given intraperitoneally in the same doses.

Guinea-pigs may receive 6 to 12 weekly intraperitoneal doses of 1 cc. each of antigens of the same concentration. One week after the sixth injection it is advisable to remove a small amount of blood from the ear of each animal for preliminary testing. If the antibody content is satisfactory the animals may be killed and the sera collected; if not satisfactory immunization should be continued and the sera retested one week after the twelfth injection.

Antigonococcus serum may be prepared by the immunization of horses and commercial antigonococcus serum employed, especially if fresh serum is obtained direct from the manufacturing laboratory.

Antituberculosis serum may be also prepared by the immunization of goats or calves with killed and finally with living bacilli.

Preparation of Antiechinococcus and Other Antiprotozoal and Antimetazoal Sera.—Rabbits or guinea-pigs may be immunized with the fluid contents of echinococcus cysts obtained from human cases or the lower animals. I believe it is better however, to grind up the cysts, add them to the fluid along with 0.5 per cent phenol or tricresol, incubate for three or four days and filter through sterile paper, the filtrate being used for immunization in the same amounts given above for bacterial antigens.

Antigens of tapeworms or other metazoa may be prepared by grinding up the parasites very thoroughly, diluting with 20 parts

by volume sterile saline solution, adding 0.5 per cent phenol or tricresol, incubating for three to five days, filtering through paper and using the filtrates.

THE PRESERVATION OF FLUID SERA FOR POSITIVE AND NEGATIVE CONTROLS.

Several methods are available but whichever is used the sera should be fresh, *unheated*, clear, free of corpuscles and preferably sterile.

They may be stored in test-tubes or ampoules with or without the addition of a preservative like 0.1 cc. of 5 per cent phenol or tricresol to each 2 cc. of serum (0.25 per cent). The containers should be kept at or near the freezing-point to prevent the development of anticomplementary properties or the loss of antibody. Frozen sera are to be preferred providing they are dispensed in ampoules containing sufficient for each day's work (0.5 or 1 cc.) and thereby avoiding the repeated freezing and thawing of larger amounts.

At ordinary refrigerator temperature of 6° to 10° C. the majority of sera ultimately become too anticomplementary for use; the method is acceptable for preserving sera for a week or two but seldom for longer periods.

The addition of preservatives tends to prevent the growth of bacteria and is advisable but due care should be exercised in their choice and the amounts used in order to avoid the anticomplementary effects of the preservative. Probably the best are 0.25 to 0.3 per cent phenol or tricresol and 50 per cent neutral chemically pure glycerin (equal parts serum and glycerin).

But even under the best of conditions sera in fluid form are apt to develop thermostabile anticomplementary properties and in my experience have proven inferior to both of the following methods employing dried sera.

DRIED SERA FOR POSITIVE AND NEGATIVE CONTROLS.

Preservation Dried in Filter Paper.—Strongly positive syphilitic sera yielding approximately 444−, or preferably 4443−, reactions in my method have been soaked into filter papers and rapidly dried at room temperature. A square 100 x 100 mm. of a fat-free paper like No. 100 W. and R. Balston for milk analysis carries from 2 to 2.5 cc.; a square of 120 by 120 mm. of ordinary filter paper or Schleich and Schull's No. 597 carries from 1.6 to 2 cc. After drying, each paper is cut into widths of 5 mm. and kept in tightly-stoppered bottles in a refrigerator.

Some of the antibody is usually lost at the end of a week; thereafter

the rate of loss is much less. For example the smallest dose of a serum yielding a ++++ reaction was 0.025 cc.; the smallest strip of paper giving a ++++ reaction was 5 x 40 mm. which carried approximately 0.04 cc. of serum; additional examples were as follows:

Smallest ++++ amount of serum.	Smallest ++++ amount of paper.
0.025 cc.	5 x 25 mm.
0.025 cc.	5 x 40 mm.
0.005 cc.	5 x 30 mm.

None of these sera were anticomplementary in dose of 0.1 cc. or in strip of 5 x 50 mm. and none of the papers acquired anticomplementary activity over a period of six months. The method therefore is usable for preserving positive and negative controls for the Wassermann reaction as stated by Hull and Faught[1] but is not a good one because of the large amounts of paper ordinarily required; a much better method is to dry 0.3 cc. of serum in small test-tubes as described below.

Furthermore the paper method is generally useless for the preservation of positive gonococcus and tuberculosis sera from human beings because the antibody content is ordinarily so low. For example, sera yielding 42− − − or weaker reactions require too much paper for a fixing dose. But when the antibody content is higher this paper method may be satisfactory. For example, the serum of a glanderous horse yielded a ++++ reaction in dose of 0.004 cc. and the paper in dose of 5 x 5 mm. (approximately 0.005 cc.) gave ++++ reactions over a period of four months when the experiment was terminated. This paper was slightly anticomplementary in dose of 5 x 20 mm. A second serum gave a ++++ reaction in dose of 0.005 cc. and the paper in dose of 5 x 6 mm. (anticomplementary in dose of 5 x 18 mm.). A third serum gave a ++++ reaction in dose of 5 x 40 mm. (0.032 cc.) and was not anticomplementary in dose of 5 x 160 mm. (0.128 cc.); four months later the smallest ++++ dose was 5 x 80 mm. (0.064 cc.) without any increase of anticomplementary activity.

The serum of a cow with contagious abortion gave a ++++ reaction in dose of 0.01 cc.; the paper gave a similar reaction in dose of 5 x 20 (approximately 0.02 cc.) and was not anticomplementary in dose of 5 x 40 mm. A second serum gave a ++++ reaction in dose of 0.0005 cc. and the paper in dose of 5 x 8 mm. (0.008 cc.); it was not anticomplementary in dose of 5 x 40 mm. Four months later the ++++ dose of serum was 0.012 cc. and of the paper 5 x 20 mm. (0.02 cc.); neither had acquired anticomplementary properties.

Normal rabbit serum may give a non-specific Wassermann reaction in dose of 5 x 30 mm. (0.028 cc.); with various bacterial antigens positive reactions may occur with 5 x 20 mm. amounts

(0.016 cc.). Therefore if rabbit immune sera are incorporated into papers for positive controls the antibody content must be sufficiently high to give ++++ reactions in smaller amounts.

A rabbit antityphoid serum gave a ++++ reaction in dose of 0.001 cc. and the paper in dose of 5 x 15 mm. (0.012 cc.); it was serviceable as a control. A second serum gave a ++++ reaction in dose of 5 x 2 mm. and was anticomplementary in dose of 5 x 12 mm. A third gave a ++++ reaction in dose of 5 x 2 mm. and was anticomplementary in only 5 x 30 mm.; both were very serviceable.

Normal horse, calf and cattle sera do not give non-specific reactions like normal rabbit sera although normal dog and mule sera may do so. A horse antigonococcus serum gave a ++++ reaction in dose of 0.05 cc. and the paper in 5 x 30 mm. (0.048 cc.); it was quite serviceable as a positive control over a period of six months. A second serum gave a ++++ reaction in 5 x 5 mm. and was anticomplementary in only 5 x 20 mm. A calf antituberculosis serum gave a ++++ reaction in dose of 0.025 cc. and the paper in dose of 5 x 40 mm. (0.032 cc.) gave similar reactions over a period of several months. A second serum gave a ++++ reaction in an amount as low as 0.0005 cc. and the paper in 5 x 4 mm. (0.0032 cc.); it was exceptionally good as likewise a third serum giving a ++++ reaction in dose of 5 x 2 mm. and anticomplementary in only 5 x 15 mm. A fourth serum was antigenic in 5 x 6 mm. and anticomplementary in 5 x 30 mm.

It is possible therefore to preserve sera containing exceptionally large amounts of antibody by this paper method for positive controls: they hold up in a refrigerator for at least four to six months and possibly longer and do not acquire anticomplementary properties. With human, horse, guinea-pig and calf immune sera the fixing dose of paper may be as much as 5 x 30 mm. but larger amounts are unsatisfactory. With rabbit, mule and dog immune sera the smallest fixing dose should not exceed 5 x 20 mm. strips in order to avoid the non-specific reactions sometimes yielded by the normal sera of these animals in my method and especially with bacterial antigens.

The technique of preparation is simplicity itself. For the Wassermann reaction the mixture of sera should give at least a 4444 − − reaction. The natural antisheep hemolysin should be removed and the *unheated* serum evenly distributed in papers measuring 100 x 100 or 120 x 120 mm. Each should be rapidly dried by pinning to a piece of unbleached muslin before a fan, cut into strips 5 mm. wide and kept in a refrigerator in tightly-stoppered bottles. At the end of a week the titer should be determined by placing increasing amounts like 5 x 5, 5 x 10, 5 x 15, 5 x 20, 5 x 25 and 5 x 30 mm. pieces in test-tubes with 1 cc. of saline solution. After allowing a few minutes for the serum to dissolve out the tubes are placed in a

water-bath at 55° C. for fifteen minutes to remove anticomplementary substances and the tests completed in the usual manner as described in Chapter XXXI by adding 10 units of antigen and 2 full units of complement followed by the primary incubation, addition of sheep corpuscles, 2 units of hemolysin and the secondary incubation. The smallest piece of paper giving a ++++ reaction is recorded as the "fixing dose" and this amount or one slightly larger may be subsequently used for positive controls. *Spinal fluids*, even from paretics, have not been generally satisfactory unless the 0.03 cc. doses gave a ++++ reaction.

An anticomplementary titration may be conducted at the same time and in the same manner except that antigen is omitted. As a general rule even pieces as large as 5 x 50 mm. are free of anticomplementary activity if the sera are fresh and properly selected.

A mixture of normal or negative sera may be prepared in the same manner and a piece equal in size to the positive control used for a negative control.

As previously stated human gonococcus, tuberculosis and typhoid sera are usually too low in antibody content for this method. The sera of glanderous horses and of horses and cows with contagious abortion may be satisfactory if they give ++++ with 0.025 cc. or similar amounts of serum.

Antigonococcus, antituberculosis, antityphoid and other immune sera prepared in rabbits may be used successfully if they contain enough antibody for their respective antigens to give ++++ reactions in 0.5 cc. of 1 to 50 dilutions (0.01 cc. serum) or preferably smaller amounts. It is hardly worth while to use weaker sera. The papers are prepared and titrated in exactly the same manner using antigen in a dose of one-third of its anticomplementary unit.

Preservation Dried in Test-tubes.—A much better method for preserving sera for positive and negative controls in the Wassermann test is as follows:

1. Select a mixture of fresh, clear sera yielding 443− −or stronger reactions.
2. Remove the natural antisheep hemolysins.
3. Distribute 0.3 cc. amounts of *unheated* serum in sterile test-tubes measuring 85 by 15 mm. as used in my test.
4. Place the open tubes in a desiccator as shown in Fig. 12 and dry as rapidly as possible . This apparatus is readily rigged up in any fairly well-equipped laboratory.
5. After drying break up the residues with a glass rod, stopper each tube tightly, paraffin the stoppers against access of moisture and keep in a refrigerator.
6. For use add 1.5 cc. of saline solution to a tube and dissolve.
7. Heat in a water-bath at 55° C. for fifteen minutes.
8. In setting up my quantitative or qualitative test this tube is

No. 1 of the series after 0.5 cc. has been removed to tube No. 2 and 0.5 cc. to tube No. 6 in the quantitative or No. 3 in the qualitative tests (the serum controls); No. 1 and the control contain the equivalent of 0.1 cc. of serum. The test is now completed in the usual manner as described in Chapter XXXI.

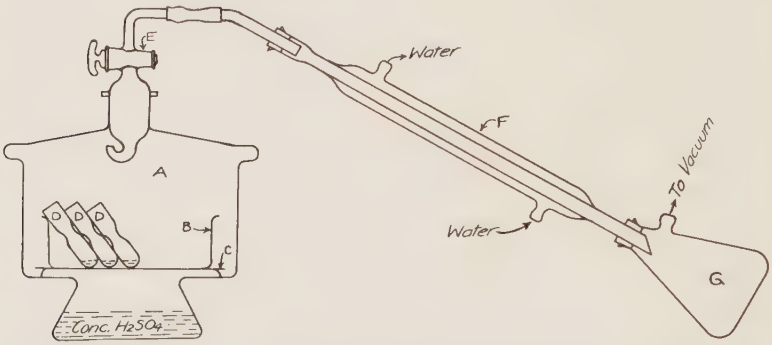

FIG. 12.—A method for drying small amounts of serum in test-tubes. A, is a vacuum desiccator containing fresh concentrated sulphuric acid and connected to a Liebig condenser F by means of a short piece of pressure tubing and a bent glass tube. The condenser is connected with a side arm suction flask to the vacuum. The drying operation is conducted as follows: The material is placed in small tubes D (if it is desired to seal the tubes these are previously contracted somewhat as shown) which are placed in an inclined position in a suitable container B on a desiccator porcelain C. With stopcock E closed the water and vacuum are turned on and stopcock E gradually opened. Care should be taken to prevent too much vacuum in the desiccator at the start as the air present in the liquid to be dried will be removed too rapidly causing frothing and loss. After all the air has been evacuated from the liquid (denoted by cessation of bubbles) stopcock E is fully opened and the vacuum maintained for about two hours or until fluid is dry, after which the stopcock is closed, the desiccator disconnected from the rest of the apparatus and the dried substance allowed to remain in the evacuated desiccator overnight. The next day the material in the test-tubes is broken up with a sterile glass rod and the tubes sealed or tightly-stoppered with sterile rubber stoppers.

Normal sera are prepared and used in the same manner for negative controls.

Almost no antibody is lost at all and anticomplementary properties are not acquired. The results observed with one positive serum serves as an example; before drying it gave a 442 — — reactions:

After drying	443 — — —
One week later	442 — — —
Two weeks later	444 — — —
Three weeks later	443 — — —
Six weeks later	432 — — —
Eight weeks later	441 — — —

The fluctuations in the third tube were probably due to slight variations in the fixability of the different complements.

If Wassermann tests are conducted twice a week it is easily possible to prepare 25 tubes (requiring 7.5 cc. of serum) at a time and sufficient for three months. I am quite sure that they will keep for over a year. Weston[2] has kept dried Wassermann positive sera for nineteen months without deterioration. Even at room temperature deterioration occurs very slowly.

The method is also admirably adapted for the preservation of positive controls of human gonococcus, tuberculosis, typhoid and other sera; also for the sera of glanderous horses and mares and cows containing contagious abortion complement-fixing antibodies.

Antigonococcus serum prepared by the immunization of horses may be used with success providing it is fresh and free of anticomplementary activity for which it should be tested before being dried. It is unnecessary to absorb with sheep corpuscles. The *unheated* serum is distributed in 0.3 cc. amounts in test-tubes, dried and stored in the same manner. For use the contents of a tube are dissolved in 1.5 cc. of saline solution, heated at 55° C. for fifteen minutes and employed as a positive control. Fresh normal horse serum may be prepared in the same manner for a negative control. The results observed with one positive serum giving a 443 — — reaction were as follows:

After drying	443 — —
One week later	443 — —
Two weeks later	442 — —
Three weeks later	443 — —
Six weeks later	443 — —

Tuberculosis sera prepared by the immunization of calves have been used in the same manner.

If an immune serum contains a very large amount of antibody smaller amounts should be dried in order to avoid using an excess of antibody. For this reason each serum should be titrated in a preliminary manner for its smallest fixing dose. If this happens to be from 0.05 to 0.005 cc. the undiluted serum may be dried in amounts of 0.3 cc. as described. If it is less than 0.005 cc. it is well to dilute the serum with an equal or more parts of saline solution before drying 0.3 cc. amounts in test-tubes.

When *rabbit* immune sera are used (gonococcus, typhoid, tuberculosis, glanders, echinococcus, etc.), the dose giving a ++++ reaction in my method should not exceed 0.025 cc. in order to avoid the non-specific reactions apt to be produced by rabbit serum with bacterial antigens. It is well to continue immunization to the point where 0.01 cc. (0.5 cc. of 1 to 50 dilution) or less gives a ++++ reaction. Therefore each serum should be titrated before drying by testing 0.5 cc. of 1 to 20, 1 to 30, 1 to 40, 1 to 50, etc., with antigen used in dose of one-third its anticomplementary unit. The serum should be previously heated at 60° to 62° C. for one-half hour to

reduce its capacity for giving non-specific reactions. If satisfactory 1 cc. of the *unheated* serum may be diluted with 9 cc. of saline solution and 0.3 cc. amounts dried in the small test-tubes. The serum need not be previously absorbed with sheep corpuscles. For use the contents of a tube should be dissolved in 1.5 cc. of saline solution and heated at 60° to 62° C. for one-half hour before being used. Each 0.5 cc. will carry 0.01 cc. of serum.

Guinea-pig immune sera do not give these non-specific reactions and undiluted serum may be dried in 0.3 cc. amounts since it is seldom that the fixing dose is less than 0.005 cc.

Larger amounts of syphilitic and other immune sera may be rapidly and satisfactorily dried by apparatus shown in Fig. 13 and adopted by Mr. Herman Brown and myself after that described

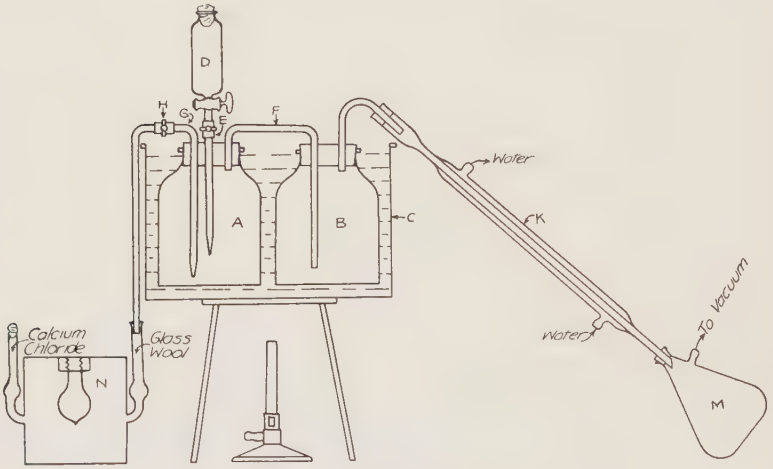

FIG. 13.—A method for drying large amounts of serum.

by Hartley, Eagleton and Okell.[3] It has worked very satisfactorily and gives a product readily and completely soluble in water or saline solution. Measured amounts of unheated serum are rapidly dried and the residue carefully weighed in order to obtain the weight of dried serum equivalent to 0.3 cc. of wet serum. For practical purposes 0.03 gm. of residue is approximately equal to 0.3 cc. serum and this amount may be weighed out and dispensed in the small test-tubes.

For use the contents of a tube may be dissolved in 1.5 cc. of saline solution or distilled water (the former being preferred in order to reduce the possible mistake of using water in the balance of the test), heated in a water-bath at 55° C. for fifteen minutes (60° to 62° C. for one-half hour if rabbit, mule or dog serum) and used as

described. Normal serum may be prepared in the same manner for negative controls.

Either method is satisfactory and I believe solves successfully the matter of providing positive and negative controls for the Wassermann and other complement-fixation tests. The sera should be selected with care on the basis of preliminary tests for fixability and freedom from anticomplementary activity and both repeated after drying. When kept in a refrigerator tightly stoppered and sealed against access of moisture, they do not acquire anticomplementary properties and the antibody undergoes but little or no deterioration following the slight initial drop at the time of drying. Each laboratory can easily maintain its own supplies and especially of positive and negative sera for the Wassermann test. Furthermore large central laboratories may easily be depots of supply at nominal cost.

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CHAPTER XXI.

INFLUENCE OF THE ORDER OF SETTING UP, TOTAL VOLUME AND SHAKING UPON COMPLEMENT-FIXATION REACTIONS.

Apparently little or no attention has been given by serologists to the possible influence upon complement-fixation of the order in which serum, antigen and complement are brought together in setting up the tests. Seligmann and Pinkus[1] have stated that the amount of complement absorbed or fixed is the same when the antigen and serum are mixed and complement immediately added or when the mixture is allowed to stand for some time before the addition of complement. Probably the majority of serologists place serum first, followed immediately by antigen, complement and saline solution.

My experiments[2] have shown that *complement should be always added last* and preferably after the serum and antigen have been mixed for five to fifteen minutes.

If antigen and complement are first mixed and allowed to stand for one-half hour before the addition of complement, the reactions are sometimes stronger than when all three are mixed in succession but these results are due to the greater anticomplementary effects of the antigen under such circumstances and consequently the antigen controls sometimes show incomplete hemolysis.

Similar results are sometimes observed when serum and complement are mixed and allowed to stand for at least one-half hour before the addition of antigen; the serum controls, however, may show slight inhibition of hemolysis due to the greater non-specific absorption of complement by serum alone under these circumstances than occurs when the three substances are mixed in succession.

When serum and antigen are mixed and allowed to stand for five to fifteen minutes before the addition of complement, the reactions are sometimes (not always) stronger than those in which serum, antigen, and complement are mixed in rapid succession and the antigen and serum controls show complete hemolysis. As pointed out by Browning and McKenzie, the mixing of serum and antigen first allows the serum to reduce the degree of anticomplementary effects of antigen. Aside from this however, I believe that the degree

of specific complement-fixation is frequently favored by the interval and especially in quantitative tests. This is shown by the results of one experiment summarized in Table 35.

TABLE 35.—THE INFLUENCE UPON COMPLEMENT-FIXATION REACTIONS IN SYPHILIS OF MIXING SERUM AND ANTIGEN FOR VARYING PERIODS OF TIME BEFORE THE ADDITION OF COMPLEMENT.

Sera.	Complement added at once.						Complement added five minutes later.						Complement added fifteen minutes later					
	0.1	0.02	0.004	0.0008	0.00016	Control.	0.1	0.02	0.004	0.0008	0.00016	Control.	0.1	0.02	0.004	0.0008	0.00016	Control.
Syphilitic	4*	4	2	—	—	—	4	4	4	—	—	—	4	4	4	—	—	—
Syphilitic	1	—	—	—	—	—	1	—	—	—	—	—	1	—	—	—	—	—
Syphilitic	4	4	1	—	—	—	4	4	4	—	—	—	4	4	4	—	—	—
Syphilitic	4	4	—	—	—	—	4	4	—	—	—	—	4	4	—	—	—	—
Syphilitic	4	4	4	1	—	—	4	4	4	1	—	—	4	4	4	2	—	—
Syphilitic	4	4	1	—	—	—	4	4	1	—	—	—	4	4	2	—	—	—
Normal	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Normal	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* 4 equals ++++; 3 equals +++; 2 equals ++; 1 equals +.

Accordingly, I believe *that something is to be gained in the way of increasing the sensitiveness of specific complement-fixation reactions by mixing antigen and serum for at least five minutes at ordinary room temperature before completing the test by the addition of complement followed by the primary incubation* and I have adopted an interval of *five to fifteen minutes* in my method.

THE INFLUENCE OF TOTAL VOLUME.

Numerous experiments have shown that the dilution of complement has an important bearing upon its hemolytic activity. In one experiment the unit of a complement diluted 1 to 30 and titrated with an antisheep system was 0.2 cc. when saline solution had been added to give a total volume of 1 to 1.5 cc., but 0.3 cc. or more when the total volume was raised to 3 cc. or higher.

Likewise in the titration of hemolysin, total volume or dilution may exert an important influence upon hemolytic activity and probably mostly upon complement; this is well shown in the results of an experiment in which the unit of an antisheep hemolysin was 0.1 cc. of 1 to 3000 in a total volume of 1 cc. but 0.1 cc. of 1 to 500 in a total volume of 5 cc.

The point of practical importance is that complement and hemolysin should be titrated in the same total volume as used in the complement-fixation tests; error may result if these are titrated in a given total volume and the units used in main tests with a higher volume.

This fact has been established in numerous experiments, the results of one being given in Table 36 as an example. In conducting this experiment the complement (diluted 1 to 30) and hemolysin were titrated in a total volume of 2 cc. and used in 2 units for comparative complement-fixation tests in which the total volume varied from 1.5 cc. to 5 cc.

TABLE 36.—THE INFLUENCE OF TOTAL VOLUME UPON COMPLEMENT-FIXATION REACTIONS IN SYPHILIS.

	Total volume 1.5 cc.						Total volume 4.5 cc.					
	0.1	0.025	0.006	0.0015	0.0004	Control.	0.1	0.025	0.006	0.0015	0.0004	Control.
Normal	−	−	−	−	−	−	+	+	+	+	+	−
Normal	−	−	−	−	−	−	+	+	+	+	+	−
Syphilitic	++	+	−	−	−	−	++	+	+	+	+	−
Syphilitic	+++	−	−	−	−	−	++++	+	+	+	+	−
Syphilitic	++++	++	−	−	−	−	++++	+++	+	+	+	+
Syphilitic	+++	++	−	−	−	−	+++	++	+	+	+	−
Syphilitic	++	+	−	−	−	−	++++	+++	+	+	+	−
Syphilitic	+++	++	+	−	−	−	++++	++++	++	+	+	−

As shown in this table, complement-fixation tests conducted with total volumes double or more than used in the titration of complement, showed incomplete hemolysis and proved unsatisfactory; the higher dilutions showed most interference with hemolysis.

In building up the technique of a complement-fixation test it is necessary to decide upon the total volume of fluid according to the depth of color desired, convenience and such factors, but it *should be a principle of technique that the complement is titrated in the same total volume (same final dilution) as used in the main complement-fixation tests;* slight differences in dilutions make no difference in the results but great differences interfere with hemolysis and particularly if the complement is highly diluted (1 to 30), as used in my method.

INFLUENCE OF SHAKING.

When serum, antigen and complement are added in setting up complement-fixation tests the mixture should be sufficiently well shaken. as likewise when corpuscles and hemolysin are added after the primary incubation. A great deal of shaking is unnecessary and may destroy a portion of complement. All that is required is a thorough mixture of the contents of each tube but foaming should be avoided. In the Kahn and other precipitation tests it has been found advisable to shake the mixtures of antigen and serum for two minutes, but

in my complement-fixation test Miss Yagle and I have observed that this practice is of no value since the shaking of serum and antigen for two minutes before the addition of complement or of serum-antigen-complement mixtures for the same interval did not increase the sensitiveness of the reactions, the results being identical with those observed when the mixtures were gently agitated for a few seconds.

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CHAPTER XXII.

QUANTITATIVE COMPLEMENT-FIXATION REACTIONS.

Since the Wassermann reaction has become widely adopted as an index of the severity of infection with *Spirochæta pallida* and as guide to treatment, a measure of the degree of complement-fixation or strength of a positive reaction with serum and cerebrospinal fluid in syphilis is generally demanded.

ADVANTAGES AND DISADVANTAGES OF A QUANTITATIVE REACTION.

It is true that from the standpoint of *diagnosis alone* it is usually sufficient to ascertain whether a serum or spinal fluid does or does not yield a positive reaction without involving the degree of positiveness; without doubt this is the more important consideration. But a *measure* of the degree of positiveness has several advantages which may be briefly enumerated as follows: (1) It gives an index of the degree of spirochetic activity although this is not necessarily parallel with the clinical state of the patient because the number and severity of lesions and symptoms may depend more upon the physiological importance of the tissues infected than upon the number and activity of organisms present. (2) It gives serological evidence of provocative or Herxheimer reactions. (3) It gives an index of the influence of specific medication and has proven of special value in estimating the comparative spirochetic activity of medicaments. (4) It enables one to chart the serological results in individual cases. (5) It increases the accuracy of serum diagnosis because tests conducted with single doses of serum like 0.1 and 0.2 cc. may occasionally give falsely negative reactions when a smaller amount like 0.05 cc. yields a true positive reaction.

The only disadvantages are the slightly greater time and larger amounts of reagents required but with a little practice in preparing the serum dilutions and especially when an automatic pipette is employed, the importance of the former is reduced to a minimum while the improved results more than justify the quantitative procedure from the standpoint of economy.

ANALYSIS OF PRESENT METHODS.

One-tube Method.—Probably the majority of serologists follow the principles of a quantitative method gradually evolved from the

work of Wassermann and Citron, that is, Wassermann's one-tube test with Citron's method of reading the reactions as strongly positive, weakly positive, doubtful and negative. Citron uses three tubes (the third being the serum control) and the same scheme for recording the reactions, but most serologists use but one dose as in Wassermann's test and Citron's scheme of recording the strength of the reaction.

Contrary to general opinion *this method is only partially quantitative.* For example, a serum may contain 4 units of syphilis antibody and give ++++ or strongly positive reaction; a second serum may contain 10 units of antibody but cannot give a result stronger than the same ++++ reaction. In other words, a certain amount of antibody is required to give a ++++ reaction in any given test conducted with a fixed amount of serum as 0.1 or 0.2 cc.; if the serum contains more than this amount of antibody, it is not shown in the reaction. Only when the amount is reduced below this amount may the reactions gradually become weaker as +++, ++, + and negative.

In practice, the serum of a patient yielding a ++++ or strongly positive reaction before treatment is instituted may continue to react in this manner for an indefinite period until the amount of antibody is reduced to the +++ level; *this may give the physician reason for suspecting that treatment is having no effect at all upon the strength of the reaction, whereas more accurate quantitative methods employing graded doses of serum or complement may show that the amount of antibody is actually being reduced.*

A 1-tube test (that is a front or antigen tube and a rear tube for the serum control) is satisfactory for determining whether or not a serum or cerebrospinal fluid contains syphilis antibody, but yields only a rough gauge of the amount of antibody. For this reason, I prefer to call it a *qualitative* rather than *a quantitative* test for the reasons given above. This test is perfectly satisfactory for determining whether or not a fluid yields a positive or negative reaction, but as a measure of the degree of positiveness it is unsatisfactory and should give way to more accurate quantitative tests.

Complement Unit Method.—Browning and McKenzie[1] titrate complement for the unit of hemolytic activity and conduct the complement-fixation test in a series of test-tubes carrying a fixed amount of serum and antigen with an increasing number of units of complement; the anticomplementary activity of each serum and the antigen are measured separately according to the number of units of complement fixed or absorbed by each and the sum of these subtracted from the number of units fixed by mixtures of serum and antigen to give the strength of the reaction.

The method is very satisfactory and with a few modifications not involving the essential principles, has yielded good results in my

hands. The main advantages are the strictly quantitative character of the test and the possibility of examining anticomplementary sera; the main disadvantages are the expense involving the use of large amount of complement serum when many sera are to be tested and difficulties in reading, permitting considerable variation due to the personal equation.

Serum Dilution Method.—Many serologists follow Citron's plan of using 2 doses of serum but as previously stated this method is only partially quantitative; Boas[2] uses a series of dilutions of serum yielding a better quantitative test.

The main advantages of a quantitative method based upon using each serum or cerebrospinal fluid in a series of amounts ranging from the largest which will "pick up" small amounts of antibody to the smallest amount at which the majority of sera containing large amounts of antibody will give a negative reaction, are those of simplicity, economy, convenience and speed with which the tests may be conducted. The main disadvantage is the possible influence of natural hemolysins in the largest amount of serum; with cerebrospinal fluid this objection does not hold and even with sera, this influence may be neutralized by easily applied technical details.

Antigen Dilution Method.—It is also possible to conduct a quantitative test by using each serum in a series of test-tubes in a fixed amount with a fixed amount of complement and varying amounts of antigen, ranging from 1 to 10 antigenic units.

I have tried this method and found it inferior to the complement unit and serum dilution methods; there are no particular advantages whereas the slight changes in antigenic activity of an extract from week to week interferes with the accuracy of results with the smaller amounts of antigen. Furthermore, it is more difficult to adjust the dose of complement to the test, whereas when one dose of antigen is employed, the complement is readily adjusted by titration in the presence of antigen. Finally, experience has shown that the strength of an antigen is not always proportional to the dose so that reactions with large amounts of antigen may be weaker than with smaller amounts.

Method Based upon Rapidity of Complement-fixation.—Complement is rapidly fixed by large amounts of syphilis antibody and antigen and Simon[3] has recently described a somewhat quantitative test based upon the principle of rapidity of complement-fixation. The objection to the method, however, is the time required when a large number of sera are to be tested at one time.

Method Proposed by the Author.—*After extensive comparative tests with all of these methods, the decision has been reached that the serum dilution method is best for a standardized technique from the standpoints of economy, accuracy and ease of manipulation.*[4]

A very large number of tests were required before a decision

could be reached regarding the proper amounts of serum and spinal fluid to use. The objects sought for in this work were as follows:

1. *To have the maximum amount of serum or spinal fluid large enough to "pick up" the smallest amounts of antibody and yet avoid non-specific reactions and particularly the anticomplementary reactions.* In my technique 0.1 cc. serum and 0.5 cc. spinal fluid were found satisfactory and equivalent approximately to 0.6 cc. serum and 3 cc. spinal fluid in terms of the original Wassermann test. Advantage has been taken therefore, of using relatively large amounts of serum and spinal fluid for increasing the delicacy of the reactions; at the same time extensive experience has proved that these amounts are perfectly safe. In an additional set of 178 comparative tests with syphilitic sera Miss Yagle and I found that an 0.2 cc. dose of serum yielded a true positive reaction when smaller amounts were negative in less than 0.2 per cent of sera and this very slight advantage was off-set by a higher incidence of anticomplementary reactions.

2. *To have the smallest amount of serum and spinal fluid in the series of dilutions small enough to give less than a ++++ reaction with sera and spinal fluids yielding very strongly positive Wassermann reactions;* under these conditions the range of doses will give a true measure of the antibody. In my test these minimum amounts have been found to be approximately 0.0025 cc. serum and 0.031 cc. spinal fluid. Indeed the great majority of syphilitic sera react in doses of 0.1, 0.05 and 0.025 cc. amounts and for this reason several serologists believe that my test could be reduced to these doses but occasionally a serum does react in the 0.0025 cc. dose and in very exceptional instances even in an amount as low as 0.0015 cc.

3. *To confine the whole range of dilution of serum and spinal fluid to 6 tubes including the serum control.* This number of tubes for each quantitative test is reasonable whereas if 8 to 12 tubes are used, the objections of excessive labor and use of materials may be raised.

The results of an extensive series of experiments have shown that a good range for sera under these conditions is 0.1, 0.05, 0.025, 0.005 and 0.0025 cc. with 0.1 cc. for the serum control as used by Detweiler[5] except that I do not use an 0.2 cc. dose of serum. As discussed more completely in Chapter XXV, it is possible to record the results with *each amount of serum* by the well known ++++, +++, ++, + and − method which gives therefore, a two-way quantitative reaction, that is, the smallest amount of serum or spinal fluid giving a positive reaction and the *degree* of positiveness with each amount. As will be noted the second tube contains one-half of the maximum amount contained in the first tube; the third tube contains one-half of the amount in the second tube while the fourth and fifth tubes contain one-fifth amounts.

With spinal fluids a range of 0.5, 0.25, 0.125, 0.0625 and 0.03125 cc.

with 0.5 cc. in the control, has been found uniformly satisfactory for a quantitative test. It is true that a few serologists have found a 1 cc. dose slightly more sensitive but Miss Yagle and I have observed that in my method there is no advantage in using this larger routinely, the results of comparative tests with 54 spinal fluids from syphilitic and non-syphilitic individuals yielding the following results:

	0.5 cc. dose.	1 cc. dose.
Per cent positive	40.7	40.7
Per cent negative	55.5	38.9
Per cent anticomplementary	3.8	20.4

The 1 cc. dose gave 7.5 per cent stronger reactions with the positively reacting fluids but in no instance yielded a positive reaction with a negative in the 0.5 cc. dose while this slight advantage was off-set by the higher percentage of anticomplementary reactions.

4. *To make these quantitative tests as economical as possible in the use of serum and spinal fluid;* only 0.3 cc. serum and 1.5 cc. spinal fluid are required and a technique has been developed whereby the range of dilutions of each serum and spinal fluid is made with rapidity and accuracy.

It should be emphasized that *these amounts of serum and spinal fluid have been found satisfactory in my particular technique;* they may not be satisfactory in other methods employing a different kind and amount of antigen, a different primary incubation and different hemolytic system.

While I originally advocated a single dose of 0.1 cc. of serum in my *qualitative* method[6] I have since adopted Sanford's practice of using 0.1 and 0.05 cc. amounts with 0.1 cc. in the serum control because the 0.05 cc. dose sometimes yields a stronger reaction than the 0.1 cc. amount and in exceptional instances a true positive reaction when the 0.1 cc. amount gives a false negative result. On the other hand the 0.05 cc. dose sometimes gives a negative reaction when the 0.1 cc. reacts positively so that both amounts are required. I have never encountered a serum reacting positively in the next smaller dose of 0.025 cc. and negatively in the 0.05 cc. amount. With spinal fluid the 0.5 cc. dose alone is sufficient for a qualitative test as the next amount of 0.25 cc. has never in my experience given a more sensitive reaction.

While it is true that 6 tubes for a test demands more labor and time than the usual 2 or 3 tubes, the actual differences in practice is slight and more than compensated by the improvement in results. As previously stated, for the purpose of determining whether or not a serum or spinal fluid does or does not give a positive reaction with a rough gauge of the degree of positiveness, the ordinary 2 or 3 tube or qualitative method is satisfactory; when a more accurate quantitative method is desired and especially as a serological guide for

treatment, the serum dilution method can be recommended and has received the endorsement of Kilduffe[7] and numerous other experienced serologists.

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CHAPTER XXIII.

INFLUENCE OF TEMPERATURE AND DURATION OF PRIMARY INCUBATION UPON COMPLEMENT-FIXATION IN SYPHILIS.

Unquestionably the two most important factors controlling the sensitiveness and specificity of all complement-fixation reactions and particularly in syphilis, are the kind and dose of antigen and method of primary incubation employed.

An analysis of the literature indicates that the majority of investigators have observed stronger Wassermann reactions when the primary incubation was conducted at a low temperature for several hours than after the usual incubation at 38° C. for one-half to one hour; this result has been generally ascribed to the greater fixation of complement by the antibody in the serum and the antigen. However, other factors require consideration before this conclusion can be accepted as proved and particularly the (*a*) influence of temperature upon complement alone and (*b*) upon the anticomplementary activity of antigen and serum. For example, slightly more deterioration of complement may occur at 8° C. for eighteen hours than at 38° C. for one hour and this would necessarily influence the results in the way of apparently stronger Wassermann reactions; or, the antigen alone and serum alone may exert more anticomplementary effects at 8° C. for eighteen hours than at 38° C. for one hour with similar results.

For the purpose of reaching a decision regarding the best method of conducting the primary incubation in the complement-fixation test for syphilis, all of these factors have required study and consideration, as likewise the velocity and amount of complement-fixation in syphilis at varying temperatures and periods of time. Our investigations have attempted to cover all of them in order to reach a conclusion on the basis of actual experimentation and experience of what constitutes the best method for conducting the primary incubation in the syphilis complement-fixation test and the results briefly summarized in this chapter have shown clearly and decisively that in tests conducted with my antigen, which contains but 0.2 per cent cholesterol in order to remove the danger of non-specific reactions, *the best method is eighteen hours in a refrigerator at 6 to 8° C. followed by ten to fifteen minutes in a water-bath at 38° C.*

Review of Literature.—Wassermann, Neisser and Bruck originally advocated placing the mixtures of serum, antigen and complement in an air

incubator for one hour; later the water-bath came into use and was particularly advocated in this country by Noguchi, an incubation of one-half hour in a water-bath at 37 to 38° C. being regarded equal to one hour in an air incubator.

In 1909 Liefmann[1] found that precipitation of chicken serum by rabbit antichicken serum and fixation of complement by the precipitates, was particularly strong after the mixtures had been kept at 0° C. for two hours. Neufeld and Haendel,[2] in a study of the influence of temperature upon complement-fixation, reached the conclusion that bacteriolytic complement was not fixed at 0° to 3° C. by cholera antigen and anticholera serum but only at 37° C., whereas hemolytic complement was fixed at both temperatures; Sachs and Bolkowska[3] also found that complement is readily fixed at low temperatures by mixtures of corpuscles and specific hemolysin.

Satta and Donati[4] studied the question of the influence of temperature upon complement-fixation in syphilis followed by Jacobstahl[5] who examined 200 sera and found 2 per cent more positive reactions with a primary incubation at 4° C. than 37° C. This report was followed by a more extensive study by Guggenheimer[6] with 623 sera tested with a primary incubation at 0° C. and 37° C.; of these sera 263 gave positive reactions at 37° C. and 267 at 0° C.; 32 sera reacted more strongly at 37° C. and negatively at 0° C. while 12 gave positive reactions at 0° C. and negative reactions at 37° C. By reason of these paradoxical results Guggenheimer suggested the advisability of testing each serum at both temperatures. Somewhat similar results were published by Altmann and Zimmern[7] who examined 1902 sera; similar reactions were observed with 1610 sera tested with a primary cold and warm incubation but 76 sera reacted positively with a cold incubation and negatively at 37° C. whereas 45 sera reacted negatively with a cold incubation and positively at 37° C. They also found that 96 sera gave stronger reactions with a cold incubation whereas 75 sera gave stronger reactions at 37° C. Later Altmann[8] studied the subject in reference to the stage of of the disease, reporting that a primary incubation at 37° C. gave a higher percentage of positive reactions with the sera of syphilitics during the primary and secondary stages of the disease whereas with sera from the tertiary and latent cases, stronger reactions occurred at 4° C. Leredde and Rubinstein[9] then published a short paper stating that best results were being observed with an ice-box period of primary incubation; Thomsen and Boas[10] also found better fixation at 16° to 18° C. than at 37° C., but Müller[11] reported best results with warm incubation at 37° C.

In this country the subject was first taken up by MacNeal[12] who tested the sera of 463 persons with a crude alcoholic extract of heart muscle; an ice-box incubation of fourteen hours at 8° to 12° C. gave 10.3 per cent more positive reactions than observed with the usual incubation of one hour at 37° C. MacNeal also tested 266 sera for gonococcus complement-fixation, finding 36.9 per cent more positive reactions with the ice-box method. As a result of these studies MacNeal believed that a period of fourteen hours at 8° to 12° C. was best for syphilis complement-fixation and six hours for gonococcus fixation. Coca and L'Esperance[13] found that some sera reacted positively only with ice-box incubation of twelve to eighteen hours, while others yielded positive reactions only at 37° C.; consequently they advised both methods for testing all sera. Kaliski then made the suggestion of combining both methods by giving a primary incubation of two hours in an ice-box followed by one-half hour in a thermostat.

Smith and MacNeal[14] have also shown an apparently important relation of the kind of extract employed as antigen to the subject of the influence of temperature upon complement-fixation; the results of their investigations may be summarized as follows: (1) The use of cholesterolized antigens in

tests with a primary incubation at 8° C. for four hours proved the most sensitive, but were also believed to yield non-specific reactions at both 37° C. and at 8° C. (2) The simple or plain alcoholic extracts used in tests incubated at 8° C. yielded more sensitive reactions than cholesterolized antigens at 37° C. and gave no non-specific reactions. (3) The acetone-insoluble lipoid extracts of Noguchi proved less sensitive than cholesterolized extracts both at 8° C. and 37° C.; they were found less sensitive than plain alcoholic extracts at 8° C. but more sensitive at 37° C. and gave no falsely positive reactions. Ottenberg[15] also reported that cholesterolized extracts gave a considerable proportion of false positive reactions with the ice-box method and believes that simple alcoholic antigens with the ice-box method of fixation (8° to 10° C.) gives by far the most delicate and accurate results.

Dean[16] has made a particularly excellent study of the influence of temperature on the fixation of complement employing normal serum and homologous antiserum and alcoholic organ extract and serum from a syphilitic patient. His experiments have shown the following: (1) Mixtures of antigen and antibody fix more complement at 0° C. than at 37° C. (2) The time which elapses before the maximum fixation of complement occurs in any mixture of antigen and antibody depends on the relative proportions of antigen and antibody; an excess of either retards the action. (3) The maximum fixation is attained more rapidly at 37° C. than at 0° C., but the amount of complement fixed is much greater at 0° C. than at 37° C. (4) Complement-fixation is due to the adsorption of euglobulin of guinea-pig serum by particles of precipitate and this adsorption is favored by keeping the mixture at a low temperature, explaining the fact that more complement is fixed at 0° C. than at 37° C.

Noguchi[17] in a study of the influence of primary incubation at 23° C., 30° C., and 37° C. upon complement-fixation with extracts of acetone-insoluble lipoids, found that complement-fixation occurs most rapidly at 37° C.; thirty minutes at 37° C. was found equal to sixty minutes at 30° C. and two hours at 23° C. irrespective of whether human or guinea-pig complement was used. Bronfenbrenner and Schlesinger[18] have also found 37° C. best for rapid fixation of complement, one-half hour incubation at this temperature being found most efficient. For complete fixation of complement incubation in an ice-box for eight to ten hours was found best, but may not be specific because fixation is so complete under these conditions "that even traces of secondary circulating antigens and their corresponding antibodies may cause fixation of complement."

Ruediger[19] found complement-fixation better in an incubator at 37° C. than in an open water-bath at the same temperature; complete fixation of complement was found to take place gradually and better at 10° C. than at 21° C. or 37° C. Incubation at 0.5° to 1° C. gave stronger fixation than at 2, 3, 4, 5, 6, 7, 8, 9, 10 and 37° C.; plain alcoholic extracts gave best fixation at 1° C. and acetone-insoluble lipoids at 37° C. Ten hours at 1° C. appeared to best meet all requirements. Larkin, Levy and Fordyce[20] also appear to favor the ice-box method, and Berghausen[21] likewise found ice-box fixation for eighteen to twenty hours at 0° to 2° C. with simple alcoholic extract of syphilitic organ, to yield better than one hour warm incubation. Owen and Martin[22] have reported that a period of four to six hours at 7° to 10° C. gave best results with simple alcoholic heart extracts. Keidel and Moore[23] observed 10.7 per cent more positive reactions among 28 cases of untreated syphilis in tests conducted by an ice-box incubation and 37.8 per cent more positives with the sera of 148 treated cases with an ice-box than with a water-bath incubation. Gilbert and Langworthy[24] found that four hours at 3° to 6° C. with cholesterolized antigen gave decidedly more sensitive reactions than one-half hour at 37° C. but since the latter sometimes

gave stronger reactions in primary syphilis it was suggested that it may be desirable to test at this temperature all sera from such cases failing to react at 3° to 6° C.

THE INFLUENCE OF PRIMARY INCUBATION UPON THE DESTRUCTION OF COMPLEMENT.

While this review of the literature is by no means complete it suffices to show that the majority of those who have investigated the subject found prolonged incubation at low temperatures (3° to 10° C.) to yield more sensitive complement-fixation reactions in syphilis than the usual one-half to one hour at 38° C. in a water-bath or thermostat. *But it is a mistake to assume that one can safely switch from the latter to the former without some changes in technique;* as a general rule, the dose of antigen and the hemolytic system must be altered in some details for satisfactory results. *One reason for this is that the temperature and duration of primary incubation have an important bearing upon the destruction or inactivation of complement entirely apart from that fixed or absorbed by antigen and antibody;* as a general rule slightly more complement must be furnished in a hemolytic system for tests conducted at 6° to 8° C. for eighteen hours than in a water-bath for one hour and for this reason I use in my test 2 *full* units instead of 2 *exact* units (the full unit being 0.05 cc. of 1 to 30 dilution more than the exact unit or amount just giving complete hemolysis).

In a study of this subject by Miss Rule and myself[25] it was found that the amount of guinea-pig complement undergoing destruction is practically identical in incubations of one-half hour water-bath and four hours at 8° C.; one hour in a water-bath resulted in slightly greater destruction than one hour in a thermostat, which probably accounts in part for the greater sensitiveness of water-bath incubation. Incubation at 6° to 8° C. for four to eighteen hours did not cause more destruction of complement than ordinarily occurred in a water-bath for one-half hour or an incubator for one hour; but when complement was titrated in the presence of antigens, the prolonged refrigerator incubation resulted in a greater degree of anticomplementary activity on the part of the extracts, which subject has also been investigated by Miss Trist and myself[26] with the following results.

THE INFLUENCE OF PRIMARY INCUBATION UPON THE ANTICOMPLEMENTARY ACTIVITY OF ANTIGENS AND SERA.

As is well known the organ extracts employed as antigens in the syphilis complement-fixation reaction may absorb or fix a slight amount of complement or actually cause the inactivation of a portion,

these effects being designated as the anticomplementary activity of antigen. To avoid non-specific complement-fixation reactions due to these factors, the antigens are titrated and employed in amounts much less than found to exert these anticomplementary effects but even these amounts are known to absorb or fix some complement, which is overcome in practice by using an excess of complement or hemolysin.

Likewise human sera frequently contain substances causing a similar non-specific fixation or inactivation of complement which are usually removable by heating at 55° C. for fifteen minutes, although they may be thermostabile and resist heating for longer periods of time. Curiously, the serum of a healthy non-syphilitic person may contain such slight amounts of these anticomplementary substances as to yield a satisfactory serum control of complete hemolysis and yet in the presence of an antigen show incomplete hemolysis, due to the summation of the so-called anticomplementary effects of serum and antigen, which separately were without demonstrable anticomplementary activity in the controls of the test.

The Influence of Primary Incubation Upon the Anticomplementary Activity of Antigens.—In conducting these experiments guinea-pig complement was titrated plain and without primary incubation by mixing varying amounts of serum with constant amounts of antisheep hemolysin and corpuscles and was given a water-bath incubation of one hour after which the unit was read.

Each organ extract was diluted 1 to 10 with physiological saline solution and used in amounts of 0.1 to 1 cc. with 2 units of complement; with each antigen and complement at least 17 sets were prepared, each set being given a different period of primary incubation before the addition of 1 unit of hemolysin and corpuscles followed by reincubation in a water-bath for one hour. The results were recorded after the tubes had stood for the settling of corpuscles.

The different antigens and primary incubations employed in these experiments are shown in the accompanying tables and figures.

Additional experiments were conducted by incubating 5 units of each antigen with increasing amounts of complement as 1, 2, 3, 4, and 5 hemolytic units; after primary incubation the amount of complement-fixation or inactivation was determined by adding 1 unit of hemolysin and sheep corpuscles and reincubating in a water-bath for one hour. The different kinds of incubation used and the results are given in Fig. 14 and may be summarized as follows:

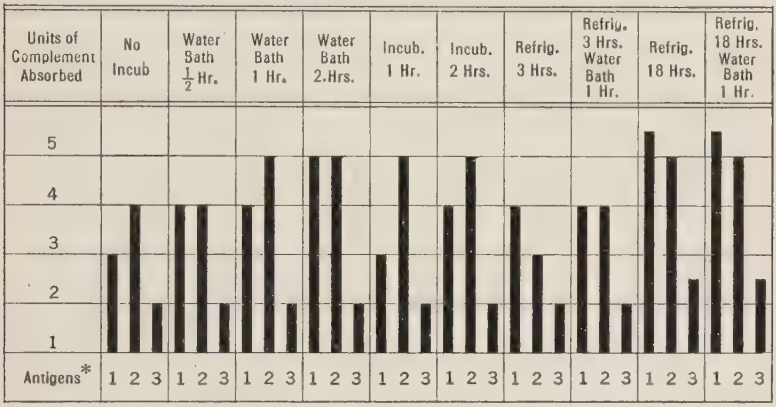
1. Temperature influences the anticomplementary activities of organ extracts differently according to the kind of extract.

2. At 37° C. the anticomplementary activity of the antigens tested was slight with a primary incubation of one-half hour, but became marked at the end of one hour and slightly more so after two hours; incubation in an open water-bath at 38° C. generally resulted in

greater non-specific fixation or inactivation of complement than in an air incubator at 37° C.

3. At 0° to 8° C. for four hours the cholesterolized and plain alcoholic extracts were practically without anticomplementary activity, although the extract of acetone-insoluble lipoids absorbed slightly more complement than at 37° to 38° C. for one hour.

4. At 8° C. for eighteen hours the cholesterolized extract and extract of acetone-insoluble lipoids become markedly anticomplementary, while the plain extracts of beef heart and syphilitic liver remain free or nearly so.


Units of Complement Absorbed
No Incub
Water Bath ½ Hr.
Water Bath 1 Hr.
Water Bath 2.Hrs.
Incub. 1 Hr.
Incub. 2 Hrs.
Refrig. 3 Hrs.
Refrig. 3 Hrs. Water Bath 1 Hr.
Refrig. 18 Hrs.
Refrig. 18 Hrs. Water Bath 1 Hr.
5
4
3
2
1
Antigens*
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3

* Antigens: 1. Cholesterolized alcoholic extract of beef heart (0.4 per cent cholesterol).
2. Plain or crude alcoholic extract of beef heart.
3. Acetone-insoluble lipoids of beef heart.

FIG. 14.—The influence of temperature and duration of primary incubation upon the anticomplementary activity of organ extracts (antigens).

5. Incubation in a water-bath at 38° C. for one-half to one hour following incubation at 8° C. for two to eighteen hours increased the anticomplementary activity of all antigens and particularly the cholesterolized extract.

These experiments show therefore, that primary incubation at 8° C. for approximately eighteen hours greatly increases the non-specific fixation of complement by antigen alone, particularly by cholesterolized extract and least by plain or crude alcoholic extracts. The stronger Wassermann reactions observed after cold incubation are due in part to this non-specific fixation and may explain the pseudopositive reactions observed by some investigators with normal sera in tests conducted with *saturated* cholesterolized extracts and incubation of about eighteen hours at 8° to 10° C. But in additional experiments it has been found that *extracts containing*

but 0.2 per cent cholesterol do not yield more non-specific reactions than plain extracts or extracts of acetone-insoluble lipoids.

The Influence of Primary Incubation Upon the Anticomplementary Activity of Heated Human Sera.—In conducting these experiments guinea-pig complement was titrated as described above and used in 2 units.

Sera of varying ages were selected, heated at 55° C. for fifteen minutes and used in amounts varying from 0.01 to 0.2 cc. with 2 units of complement; these mixtures were kept at varying temperatures for varying periods of time when 1 unit of hemolysin and sheep corpuscles were added followed by reincubation in a water-bath for one hour to detect the effects of the sera upon complement according to the degree of hemolysis.

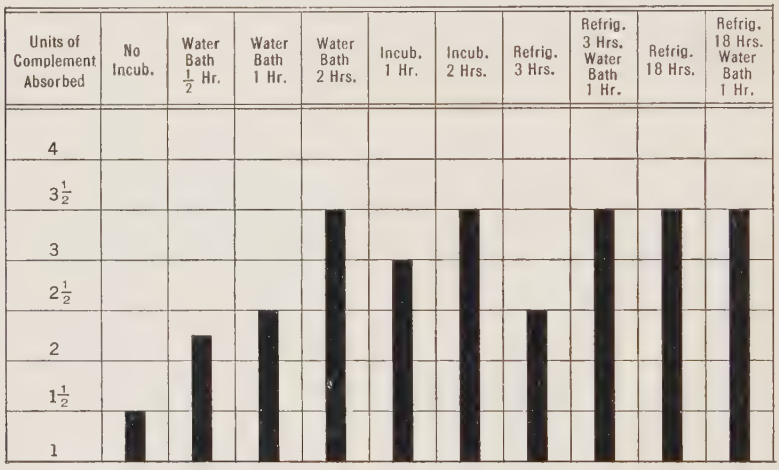


FIG. 15.—The influence of temperature and duration of primary incubation upon the anticomplementary activity of human sera.

Additional experiments were conducted by using heated sera in constant amounts of 0.1 cc. with increasing amounts of complement as 1, 2, 3, and 4 units; after primary incubation 1 unit of hemolysin and sheep corpuscles were added followed by reincubation in a water-bath for one hour. A summary of the results observed with three sera are shown graphically in Fig. 15, indicating the influence of temperature and duration of primary incubation upon the absorption or fixation of complement by sera.

As expected, the results varied according to the freshness of the sera and the presence or absence of thermolabile and thermostabile antilysins; also according to the amounts of natural antisheep hemolysin present in each serum. The majority of perfectly fresh sera yielded entirely negative results, that is, were free of anticomplemen-

tary activity; older sera and particularly those containing bacteria showed varying effects upon complement.

The general results of these experiments have shown the following:

1. Incubation of serum and complement at 38° C. for one hour and particularly in a water-bath may result in the inactivation or non-specific fixation of considerable complement; the amount of this non-specific fixation or inactivation is still greater after two hours' incubation.

2. *At 6° C. for four to eighteen hours there is practically no fixation or inactivation of complement by serum alone; heat, especially in the water-bath at 38° C., favors this phenomenon in a special manner.* For this reason when sera are placed in a water-bath at 38° C. for an hour after a primary incubation at 8° C. for four to eighteen hours, a portion of the complement is fixed or inactivated by the antilysins of the serum.

THE INFLUENCE OF PRIMARY INCUBATION UPON THE VELOCITY AND AMOUNT OF COMPLEMENT FIXATION IN SYPHILIS.

But aside from these non-specific effects, it is possible that more complement is specifically fixed by antigen and antibody by prolonged cold incubation than in the usual warm incubation at 38° C. for one hour; however, the period of cold incubation greatly prolongs the time required for conducting diagnostic tests, so that it is a matter of considerable practical importance to determine by comparative tests whether shorter periods of primary incubation may not yield similar results.

With these objects in view Miss Rule, Miss Yagle and myself[27] have studied the velocity and amount of complement-fixation in syphilis with different antigens at different temperatures, as bearing upon the choice of a primary incubation for the most sensitive reactions consistent with specificity.

The Velocity of Complement-fixation in Syphilis at Varying Temperatures.—The results of experiments conducted with syphilitic sera and various antigens at 38° C. were as follows:

1. Complement-fixation with strongly syphilitic sera used undiluted in amount of 0.1 cc. is frequently very rapid and may occur within a few minutes at room temperature (20° to 23° C.) with no primary incubation at all; this is especially true when cholesterolized extracts and extracts of acetone-insoluble lipoids are employed. Incubation at 38° C., however, usually increases the degree of complement-fixation.

2. Complement-fixation is usually but not always more rapid in a water-bath at 38° C. than in an air incubator at the same temperature, as determined by comparative tests with incubations varying

from fifteen minutes to two hours. This is true with both plain and cholesterolized extracts. *With these antigens, however, one-half hour in a water-bath is scarcely equal to one hour in the incubator, as is commonly stated to be the case.*

3. Incubations at 38° C. for more than two hours are frequently unsatisfactory with these conducted with 2 units of complement because sufficient inactivation or destruction of complement occurs under these conditions to produce incomplete hemolysis of the serum and antigen controls.

4. The velocity of complement fixation in syphilis at 38° C. varies with the kind of antigen employed, being most rapid with cholesterolized extracts and least with plain or crude extracts.

5. *In general terms complement-fixation reaches the maximum degree at 38° C. in a water-bath with extracts of acetone-insoluble lipoids in from thirty to forty-five minutes; with cholesterolized extracts in one to two hours; and with plain or crude alcoholic extracts at least two to three hours are required.*

Experiments conducted with primary incubations at 0° to 15° C. yielded the following results:

1. Complement-fixation at low temperatures (0° to 8° C.) is surprisingly rapid, well-marked reactions being observed even after incubations of but fifteen minutes.

2. Complement-fixation in syphilis occurs somewhat more rapidly at 8° C. than at 0° to 2° C.; in general terms four hours at 2° C. yields the same or slightly greater fixation of complement than occurs in two hours at 8° C.

3. The optimum temperature for complement-fixation in syphilis with cold incubation is from 6° to 15° C.; with a period of three hours, more complement is fixed at 2° C. than at 0° C. and more at 6° C. than at 2° C.; at 6° to 15° C. there is practically no difference in the degree of fixation, although at 20° C. (room) slightly stronger reactions are observed which, however, are frequently due to destruction or inactivation of complement rather than specific fixation of complement.

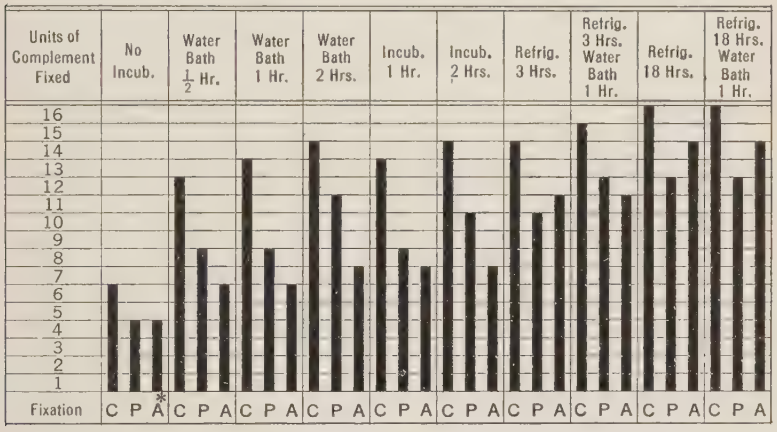
4. *Water-bath incubation at 38° C. for one-half to one hour results in the fixation of more complement than at 2° to 8° C. in three hours but less than occurs at 8° C. for three hours plus one hour in a water-bath and much less than at 2° to 8° C. for eighteen hours. This is apparently true with both plain and cholesterolized extracts and extracts of acetone-insoluble lipoids.*

THE INFLUENCE OF PRIMARY INCUBATION UPON THE AMOUNT OF COMPLEMENT FIXED BY SYPHILITIC SERA AND ANTIGEN.

By reason of the influence of temperature and duration of primary incubation upon complement alone and more especially upon the

anticomplementary effects of organ extracts and sera alone as summarized above, the technique of Browning and McKenzie has been found very useful for determining the influence of temperature upon the fixation of complement by mixtures of syphilitic serum and extract; with a modification of this technique the amount of complement fixed by antigen alone and serum alone has been measured in units and subtracted from the number of units fixed by the mixture of the two.

The results of one experiment with a syphilitic serum and three different organ extracts are shown in Fig. 16, that is, the amounts of complement expressed in units fixed by serum and antigen minus that fixed by these separately, are given for each of eight different primary incubations.



C = cholesterolized extract; P = plain extract; A = acetone-insoluble lipoids.

FIG. 16.—The influence of temperature and duration of primary incubation upon the fixation of complement measured in units of syphilitic serum and three different antigens.

The results of these experiments have shown:

1. With cholesterolized extracts complement-fixation occurs more rapidly and to greater degree at all temperatures than with plain extracts; cold incubation is especially favorable to complement-fixation by plain alcoholic extracts and extracts of acetone-insoluble lipoids.

2. Cold incubation at 8° C. for three to eighteen hours increases the amount of complement absorbed or fixed by mixtures of syphilitic serum and antigen; the stronger complement-fixation reactions observed with all antigens at 8° C. are due not only to an increase of complement-fixation by serum alone and antigen alone, but also to an actual increase of specific fixation by mixtures of these.

THE KIND OF ANTIGEN IN RELATION TO PRIMARY INCUBATION IN COMPLEMENT-FIXATION IN SYPHILIS.

The amount of complement fixed or absorbed by organ extracts alone varies therefore with the kind of extract employed. As a general rule alcoholic extracts containing 0.4 per cent cholesterol absorbed or fixed more complement at different temperatures and particularly at 8° C. for eighteen hours than plain extracts and extracts of acetone-insoluble lipoids.

MacNeal and others have found that cholesterolized extracts are most sensitive both at 8° and 38° C., but plain extracts are as sensitive as cholesterolized extracts when the primary incubation is conducted at 8° C. and that non-specific reactions do not occur. Extracts of acetone-insoluble lipoids were found inferior in antigenic sensitiveness to cholesterolized extracts both at 38° and 8° C.; compared with plain alcoholic extracts they were found inferior at 8° C. but superior at 38° C.

In other words while temperature influences the anticomplementary activity of an extract, it also appears to influence the antigenic sensitiveness, that is, an extract will apparently fix more complement with a syphilitic serum at one temperature than at another, the degree of anticomplementary activity of antigen and serum alone being constant. This is well shown in Fig. 16 and in the results of additional experiments summarized as follows:

1. With a primary incubation in an incubator or water-bath at 38° C. for one-half to two hours, complement-fixation occurs much more rapidly and completely with cholesterolized extracts and acetone-insoluble lipoids than with plain extracts of heart muscle and syphilitic liver.

2. With primary incubation at 8° C. the degree of complement-fixation with the four different antigens is more nearly equal, that is, the antigenic sensitiveness of the plain extracts is increased to a much greater degree than the cholesterolized extracts and extracts of acetone-insoluble lipoids; in incubations at 8° C. for sixteen to twenty-four hours the antigenic sensitiveness of plain extracts of heart muscle and syphilitic liver "pick up" in a remarkable manner, but even at best are not quite equal in antigenic activity to cholesterolized extracts.

3. In primary incubations of four hours at 8° C. plus one hour in the water-bath at 38° C. plain extracts are much inferior in antigenic sensitiveness to cholesterolized extracts and acetone-insoluble lipoids.

4. *Incubations of one hour in a water-bath at 38° C. do not result in the fixation of as much complement as in incubation of three hours at 8° C. plus on hour at 38° C. or as in incubation of eighteen hours at 8° C. This is true of all antigens but especially in the case of plain extracts.*

THE AUTHOR'S METHOD OF PRIMARY INCUBATION IN COMPLEMENT-FIXATION TESTS FOR SYPHILIS.

The ideal period of primary incubation is that which results in the least destruction of complement, the least non-specific fixation of complement and *the maximum specific fixation;* a search for this involves a consideration of the kind of antigen employed and of practical working conditions, that is, the method must be applicable to the routine examination of large numbers of sera with a minimum of labor and time.

Aside from these considerations there remains the important one concerning the possibility of the existence of one kind of syphilis antibody fixing complement best at 38° C. and a second at lower temperatures; if these exist, the best complement-fixation work is only possible by conducting duplicate tests with each serum as suggested by Guggenheimer and by Coca and L'Esperance or, by a combined primary incubation of some hours at 8° to 10° C. followed by a period at 38° C., as suggested by Kaliski.

In all of the comparative complement-fixation tests conducted by Rule, Trist and myself[28] with a primary incubation at 38° C. for one hour and a second set at 8° to 10° C. for about eighteen hours, using the same sera, antigens and general technique, no evidence was obtained indicating the existence of two kinds of antibody, differences were encountered and especially with weakly positive sera, but when the same technique was employed *the only differences encountered were positive reactions with some sera tested at 8° C. which reacted negatively at 38° C. and stronger reactions* at *8° C. than at 38° C.*

In other words *a percentage of weakly positive sera and especially those from treated cases of syphilis, will yield negative reactions when tested at 38° C. for one hour and positive reactions when tested at 8° C. for four to eighteen hours.* This is especially true if a plain antigen is employed; with cholesterolized extracts the differences are much less, because cholesterolized extracts absorb or fix complement more rapidly at all temperatures and especially at *38°* C. It is true, however, that we may not have tested a sufficient number of sera from cases of *primary syphilis* since Gilbert and Langworthy believe that in this stage incubation at 38° C. may be more sensitive than at 6° to 8° C. for eighteen hours. All that I may state at present is that I have not yet encountered the phenomenon in tests by my method and antigen but in view of the above statements I adopted in 1921 an incubation of sixteen to eighteen hours at 6° to 8° C. *followed by ten to fifteen minutes in a water-bath at 38° C.* Certainly in tests employing my technique and antigen the reactions appeared to be the most sensitive obtainable within the confines of specificity and the work of testing a large number of sera is facilitated by dividing

it into two days instead of crowding it into one, as is necessary with a primary incubation of three hours at 8° C. followed by one hour at 38° C. or of four hours in a refrigerator. The differences are not so great, however, but that the latter method may be employed if found more convenient. If a refrigerator is not available a temperature of 8° C. is readily secured in an open pan of water and ice for a period of three or four hours. A primary incubation of eighteen hours at 8° to 10° C. is best secured in an ordinary ice or electric refrigerator; *the temperature should not be below 6° C. or higher than 10° C. for best results.*

During the past six years my assistants and I have repeated the experiments many times in the hope of finding a shorter method but always with the same general result, namely, that with my method and new antigen the most sensitive reactions were obtained with an incubation of eighteen hours at 6° to 8° C. followed by ten to fifteen minutes at 38° C.

In one set of comparative tests with 100 sera of syphilitic and non-syphilitic origin set up at the same time with the same antigen in the same dose and same hemolytic system the results were as follows:

TABLE 37

Incubation.	Positive, per cent.	Negative, per cent.	Anticomplementary, per cent.
(*a*) Eighteen hours refrigerator and ten minutes water-bath	63	35	2
(*b*) Four hours refrigerator and ten minutes water-bath	58	39	3
(*c*) Three hours refrigerator and one hour water-bath	54	37	9

All of the positive reactions were from syphilitic cases, the majority under treatment. Doubtless many of the 9 per cent listed as anticomplementary in the method employing three hours in the refrigerator followed by one hour in the water-bath were not really due to an anticomplementary state of the sera but rather to an increased destruction of complement resulting in incomplete hemolysis of the serum controls.

In a second set of similar comparative tests with 249 syphilitic and non-syphilitic sera essentially similar results were observed as follows:

TABLE 38

Incubation.	Positive, per cent.	Negative, per cent.	Anticomplementary, per cent.
(*a*) Eighteen hours refrigerator and ten minutes water-bath	50	47	3
(*b*) Two hours refrigerator and one hour water-bath	36	63	1
(*c*) Two hours water-bath	38	46	16

Kilduffe[29] has reported similar results in comparative tests with 300 sera employing my antigen and quantitative method as follows:

TABLE 39

Incubation.	Positive, per cent.	Negative, per cent.	Anticomplementary. per cent.
Sixteen hours ice-chest	35.3	60	1.6
Four hours ice-chest	29.1	69	1.0
Four hours ice water-bath	30.0	69	1.0

All of the positive reactions observed in my series were specific reactions and I believe the same was true of the series reported by Kilduffe and leading to the conclusion that prolonged incubation at low temperatures results in the largest number of true positive reactions.

Duke[30] has attempted to shorten the period by the following method: "The tubes are placed in a rectangular galvanized iron pan with stopcock for drainage. Ice-water is poured into the pan until it reaches a level higher than that of the fluid in the tubes. The temperature of this water is kept at 8° C. for fifteen minutes by the addition of small pieces of ice. (The temperature of a tube placed in the ice-water-bath is reduced to 9° C. in five minutes or less). After the expiration of fifteen minutes, the ice is removed with the result that the temperature of the ice-water-bath rises approximately 1° each half hour. At the end of the hour, the ice-water is drawn off by opening the stopcock and the pan is refilled with water at 40° C. The tubes are allowed to remain in this for a period of five to ten minutes in order to remove the chill from tubes before they are placed in the 37.5° C. water-bath. The sensitized cells are then added and the tubes are placed in the 37.5° C. water-bath for one hour at which time the readings are made. The above details are important. If the tubes are not warmed before they are placed in the warm-water-bath, the warm-water-bath is cooled down with the result that the complement acts too slowly and may make the control tubes simulate the reaction of an anticomplementary serum."

He reports that this method has given results equal to an incubation of four hours in an ice-box. This may be true but in the comparative tests by Miss Yagle and myself it was found to yield from 8 to 10 per cent fewer true positive reactions than duplicate tests conducted by an incubation in a refrigerator at 6 to 8 ° C. followed by ten to fifteen minutes in a water-bath at 38° C. In one series of tests the latter yielded 39.2 per cent true positive reactions and the former 31.4 per cent., in a second series the latter yielded 41 per cent true positive reactions and the former 31 per cent, the number of anticomplementary reactions being almost identical in both methods (1 to 2 per cent).

Therefore I have found no escape from the conclusion that in syphilis complement-fixation tests employing an antigen containing not more than 0.2 per cent cholesterol, the most sensitive and specific reactions have occurred with a primary incubation of sixteen to eighteen hours at 6° to 8° C. followed by ten to fifteen minutes in a water-bath at 38° C.; certainly this is true in the case of my hemolytic system. It necessarily prolongs the time required for rendering reports, but from the technician's standpoint it divides the work between two days and enables more tests to be conducted. From the clinician's standpoint it means greater sensitiveness within the limits of specificity and certainly there can be no more important consideration.

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CHAPTER XXIV.

INFLUENCE OF TEMPERATURE AND DURATION OF PRIMARY INCUBATION UPON BACTERIAL COMPLEMENT-FIXATION REACTIONS.

Following the usual custom, complement-fixation tests in bacterial infections as tuberculosis, typhoid fever, glanders and gonorrhea are usually conducted with a primary incubation of one hour in a water-bath or air incubator. As reviewed in the preceding chapter numerous investigations with the syphilis complement-fixation test have shown, however, that stronger and more sensitive reactions are generally observed when refrigerator incubation is employed for longer periods.

When a large amount of antibody is present in a serum as during the acute stages of syphilis, complement-fixation occurs rapidly at all temperature from 6° to 38° C. but in most bacterial infections there are relatively small amounts of complement-fixing antibody which increases the importance of the kind and duration of primary incubation employed in the interests of the most sensitive reactions.

INFLUENCE OF THE KIND AND DURATION OF PRIMARY INCUBATION UPON THE ANTICOMPLEMENTARY ACTIVITY OF BACTERIAL ANTIGENS.

Rule, Trist and I have titrated guinea-pig serum complements in the presence of tuberculosis, typhoid, glanders and gonococcus antigens with different kinds of primary incubation to determine the influence of kind and duration of primary incubation upon the destruction of complement and non-specific fixation of complement by these antigens.

Each bacterial antigen was used in one-quarter the anticomplementary unit (titrated in a water-bath) with amounts of 1 to 30 dilutions of mixed guinea-pig serum complements varying from 0.15 to 0.5 cc. The following primary incubations of complement and antigen were employed before the addition of 2 units of hemolysin and sheep corpuscles followed by a secondary incubation of one hour in a water-bath:

One hour in an air incubator (38° C.)
One hour in a water-bath (38° C.)
Two hours in a water-bath (38° C.)
Four hours in a refrigerator (6° to 8° C.)
Eighteen hours in a refrigerator (6° to 8° C.)

With all of the bacterial antigens employed in this study primary incubation of antigen and complement for one hour in a water-bath resulted in slightly more fixation of complement than after one hour in an incubator; two hours in a water-bath resulted in still greater fixation of complement. Four hours in a refrigerator gave about the same fixation as one hour in a water-bath while eighteen hours in a refrigerator usually resulted in slightly greater fixation than with one hour in a water-bath but less than occurred with two hours in a water-bath. For example, complement titrated in the presence of a tuberculosis antigen yielded the following units of complement diluted 1 to 30:

0.25 cc. with a primary incubation of one hour incubator.
0.3 cc. with a primary incubation of one hour water-bath.
0.4 cc. with a primary incubation of two hours water-bath.
0.3 cc. with a primary incubation of four hours refrigerator.
0.35 cc. with a primary incubation of eighteen hours refrigerator.

Fig. 17 gives the results of titrations of various bacterial antigens for anticomplementary activity; each antigen was employed in 0.5 cc. of varying dilutions with 2 units of complement. Antigen and complement were incubated by three different methods, namely, one and two hours water-bath and eighteen hours refrigerator, before the addition of hemolysin and corpuscles.

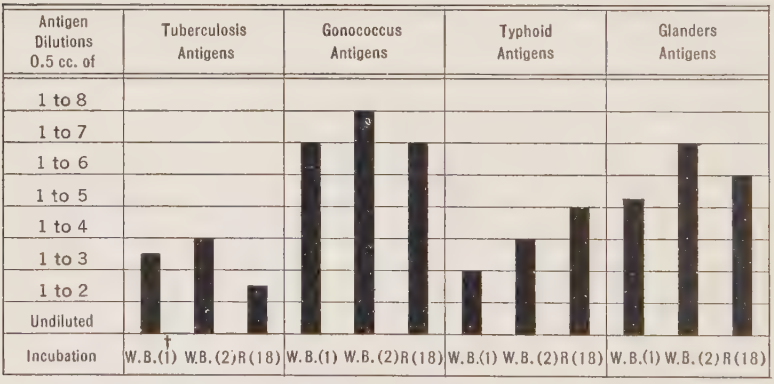


Fig. 17.—The influence of primary incubation upon the anticomplementary activity of bacterial antigens.*

* Average results with 12 different antigens of each bacterium.

† One hour in water bath at 38° C.; two hours in waterbath; R = refrigerator for sixteen to eighteen hours at 6° to 8° C.

The anticomplementary activity of the majority of antigens was least after a primary incubation of one hour in a water-bath but highest after two hours incubation; after a primary incubation of

eighteen hours in a refrigerator the anticomplementary activity was the same or but slightly greater than after one hour water-bath. The results observed with glanders antigen are good examples, the anticomplementary units being as follows:

0.5 cc. of 1 to 5 after primary incubation of one hour water-bath.
0.5 cc. of 1 to 7 after primary incubation of two hours water-bath.
0.5 cc. of 1 to 6 after primary incubation of eighteen hours refrigerator.

The results of all experiments may be briefly summarized by the statement that *heat especially favors the non-specific fixation of complement by bacterial antigens and particularly of antigens of tubercle bacilli; cold especially favors non-specific fixation by alcoholic tissue extracts.*

THE INFLUENCE OF KIND AND DURATION OF PRIMARY INCUBATION UPON BACTERIAL COMPLEMENT-FIXATION REACTIONS.

Numerous experiments have been conducted with antigens of gonococci, typhoid, glanders and tubercle bacilli and *B. abortus* of cattle, to determine the influence of kind and duration of primary incubation upon bacterial complement-fixation. In conducting these experiments each antigen was employed in an amount equivalent to one-quarter its anticomplementary unit with varying amounts of immune sera. Most of the experiments were conducted with the following primary incubations:

One hour in a water-bath at 38° C.
Two hours in a water-bath at 38° C.
Eighteen hours in a refrigerator at 6° to 8° C., followed by ten minutes in a water-bath at 38° C.

For the sake of brevity the results have been summarized in the form of graphic charts showing the least amounts of the respective immune sera yielding ++++ reactions with the different antigens and primary incubations. In all instances the serum, antigen and hemolytic controls showed complete hemolysis.

Fig. 18 gives the results of two experiments with rabbit immune sera and gonococcus antigen and shows that the strongest reactions were observed with refrigerator incubation of eighteen hours. For example, a ++++ reaction was observed with 0.5 cc. of 1 to 80 dilution of rabbit serum No. 7 with a primary incubation of one hour in a water-bath; with 0.5 cc. of 1 to 120 dilution after two hours in a water-bath, but with 0.5 cc. of as high a dilution as 1 to 225 when the primary incubation was eighteen hours at 6° to 8° C. followed by ten minutes in a water-bath.

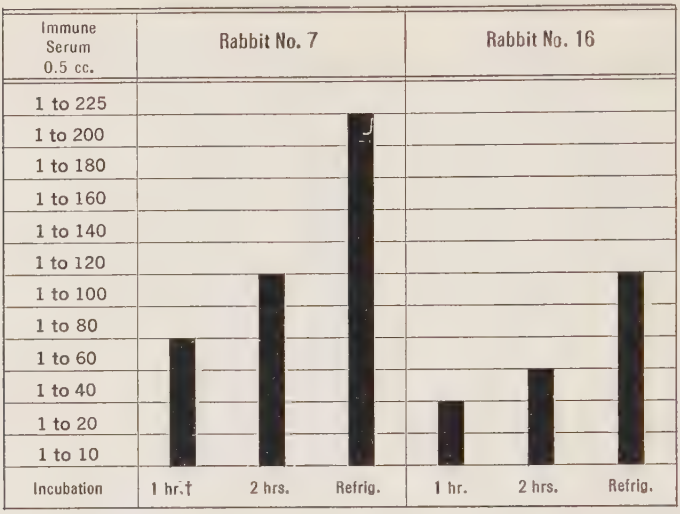


FIG. 18.—The influence of primary incubation upon the *gonococcus* complement-fixation reaction.*

* Average of results observed with 22 antigens and 2 rabbit immune sera.

† One hour in water bath at 38° C.; two hours in water bath at 38° C.; sixteen to eighteen hours in refrigerator at 6° to 8° C.

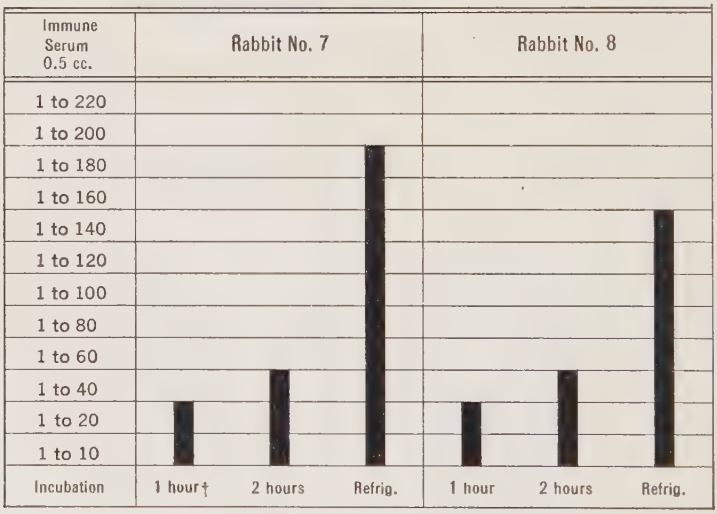


FIG. 19.—The influence of primary incubation upon the *typhoid* complement-fixation reactions.*

* Average of results observed with 24 antigens and 2 rabbits immune sera.

† One hour in water bath at 38° C.; two hours in water bath at 38° C.; sixteen to eighteen hours in refrigerator at 6° to 8° C.

Fig. 19 summarizes the results observed with two rabbit anti-typhoid sera and typhoid antigen and shows that the strongest reactions were observed with a refrigerator incubation of eighteen hours. For example, ++++ reactions were observed with 0.5 cc. of 1 to 40 and 1 to 60 dilutions of a rabbit immune serum with primary incubations of one and two hours in a water-bath, but with a dilution as high as 0.5 cc. of 1 to 200 with cold incubation of eighteen hours followed by ten minutes in a water-bath.

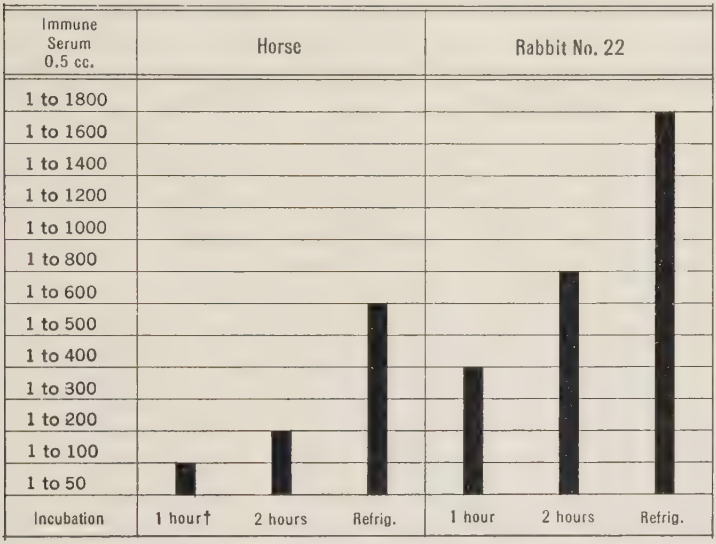


FIG. 20.—The influence of primary incubation upon the *glanders* complement-fixation reactions.*

* Average of results observed with 24 antigens and 2 immune sera (horse with glanders and immunized rabbit).

† One hour in water bath at 38° C.; two hours in water bath at 38° C.; sixteen to eighteen hours in refrigerator at 6° to 8° C.

Fig. 20 gives the results observed with glanders antigen and two immune sera. With the serum of a glanderous horse ++++ reactions were observed with 0.5 cc. of 1 to 100 and 1 to 200 dilutions with primary incubations of one and two hours in a water-bath, but with a 0.5 cc. of 1 to 600 with a refrigerator incubation of eighteen hours followed by ten minutes in a water-bath.

Fig. 21 shows the results observed with the serum of a cow infected with *B. abortus* (Bang), and an antigen of this bacillus kindly furnished by Dr. Fred Boerner. With warm incubations of one and two hours, ++++ reactions were observed with 0.5 cc. of 1 to 80 to 1 to 320 dilutions of immune serum, but with refrigerator incubation of eighteen hours followed by ten minutes in a water-bath,

++++ reactions were observed with dilutions as high as 1 to 2560.

Fig. 22 summarizes the results observed with sera from human subjects with active pulmonary tuberculosis. The titrations were made with one of Petroff's antigens.

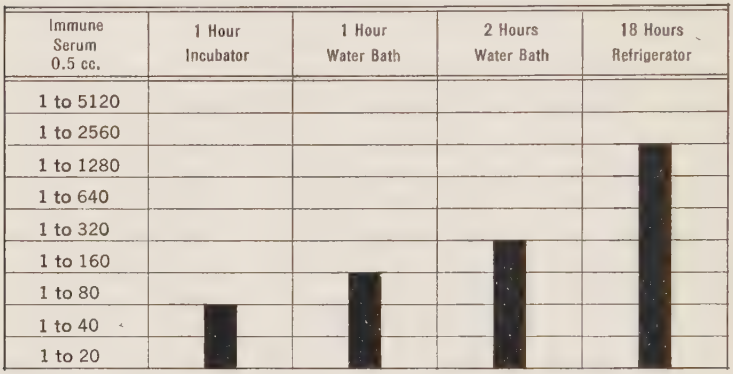


FIG. 21.—The influence of primary incubation upon the *contagious abortion* complement-fixation reaction.

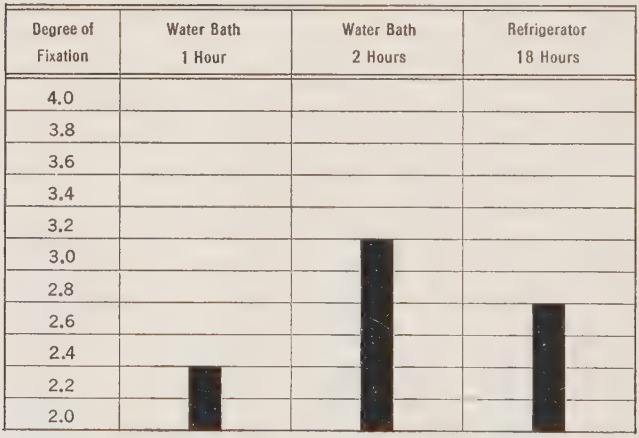
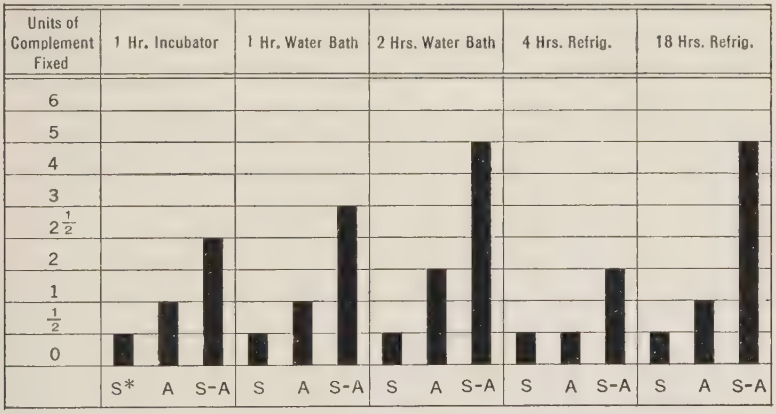


FIG. 22.—The influence of primary incubation upon the *tuberculosis* complement-fixation reaction with human sera.

Curiously, slightly stronger reactions were observed with a primary incubation of two hours in a water-bath than with any other kind of primary incubation. As previously stated tuberculosis antigens were found to be most anticomplementary with water-bath incubation and I believe that the stronger complement-fixation reactions may be explained on this basis.

To make sure that this deduction was correct, additional experiments were conducted by using each antigen in one-quarter its anticomplementary unit with a fixed amount of immune serum and an increasing number of units of complement to determine how many units of complement were fixed or absorbed by serum alone, antigen alone and by serum and antigen. Each experiment was conducted with five different primary incubations and the results observed with a tuberculosis antigen and a calf immune serum are shown in Fig. 23. With a primary incubation of two hours in a water-bath the serum and antigen alone showed a combined non-specific fixation of 2.5 units of complement; the mixture of serum and antigen fixed 5 units showing therefore a specific fixation of 2.5 units of complement. In the primary incubation of eighteen hours at 6° to 8° C. followed by ten minutes in a water-bath, serum and antigen alone showed a combined non-specific fixation of 1.5 units of complement; the mixture of serum and antigen fixed 5 units showing therefore a specific fixation of 3.5 units of complement.



* S = serum alone; A = antigen alone and S–A = serum and antigen.

FIG. 23.—The influence of kind and duration of primary incubation upon specific and non-specific *tuberculosis* complement-fixation tests.

Similar experiments were conducted with antigens of typhoid and glanders bacilli and the results have demonstrated quite conclusively that primary incubation of eighteen hours at 6° to 8° C. followed by ten minutes in a water-bath greatly favors and aids the specific fixation of complement by bacterial antigens and their respective immune sera, although warm incubation may favor in higher degree non-specific fixation by antigen alone and particularly by antigens of tubercle bacilli. In preparing Table 40 the amounts of complement fixed non-specifically by antigens alone and sera

alone were subtracted from that fixed by mixtures of sera and antigen, the differences giving the amounts of complement fixed specifically.

TABLE 40.—UNITS OF COMPLEMENT *Specifically* FIXED IN VARIOUS BACTERIAL COMPLEMENT-FIXATION REACTIONS DURING DIFFERENT PRIMARY INCUBATIONS.

Tests.	1 hour incubator.	1 hour water-bath.	2 hours water-bath.	4 hours refrigerator.	18 hours refrigerator.
Tuberculosis	1	1½	2½	1	3½
Typhoid	1	1½	2	½	5
Glanders	½	½	1	1	3

Specific complement-fixation by all of the bacterial antigens and respective immune sera employed in our studies (tuberculosis, glanders, typhoid, contagious abortion and gonococcus) was greatly increased therefore by a refrigerator incubation of eighteen hours at 6° to 8° C. followed by water-bath incubation of ten minutes. Water-bath incubation of two hours may show slightly stronger tuberculosis complement-fixation reactions owing to the greater non-specific fixation by antigen alone, but cold incubation of eighteen hours favors a greater degree of specific fixation.

Accordingly, I have reached the conclusion that *for all bacterial complement-fixation tests* primary incubation should be eighteen hours in a refrigerator at 6° to 8° C. followed by ten to fifteen minutes in a water-bath, as used in tests for syphilis.

CHAPTER XXV.

INFLUENCE OF TEMPERATURE AND DURATION OF PRIMARY INCUBATION UPON COMPLEMENT-FIXATION REACTIONS FOR THE IDENTIFICATION OF BLOOD STAINS AND OTHER PROTEINS.

While the precipitin test has been usually employed for the identification and differentiation of such substances as serum, blood stains, meat extracts, milk, semen, etc., yet the complement-fixation test has proven more sensitive and generally satisfactory as reviewed in Chapters L, LI, LII and LIII.

But the sensitiveness of the reactions is likewise greatly influenced by the kind of primary incubation employed and for this reason has been given the extensive study by Rule and myself that its practical importance demands. The following antigens and antisera were employed:

1. Extracts of human blood stains on cloths tested with rabbit and guinea-pig antihuman sera. (2) Extracts of beef blood stains on cloths tested with rabbit and guinea-pig antisera. (3) Filtered and sterilized cow's milk tested with guinea-pig anticow sera. (4) Saline extracts of sheep flesh tested with rabbit antisheep sera. (5) Saline extracts of dog flesh tested with rabbit antidog sera.

Additional antigens of cooked dog and sheep flesh and of seminal stains with their corresponding antisera have been employed, but the results of complement-fixation reactions are given in Chapters LI and LII on comparative studies of the precipitin and complement-fixation reactions for the identification and differentiation of soluble proteins. The following periods of primary incubation were employed:

One hour in an incubator at 38° C.
One hour in a water-bath at 38° C.
Two hours in a water-bath at 38° C.
Four hours in a refrigerator at 6° to 8° C.
Eighteen hours in a refrigerator plus ten minutes in a water-bath.

THE INFLUENCE OF KIND AND DURATION OF PRIMARY INCUBATION UPON THE ANTICOMPLEMENTARY ACTIVITY OF EXTRACT ANTIGENS.

Warm incubation favors the non-specific fixation of complement by extracts of blood stains, sera, milks and extracts of meats more than cold incubation; the results observed with an extract of human

blood stain and a saline extract of uncooked dog flesh are shown in Fig. 24 as general examples of the results observed in these antigen titrations.

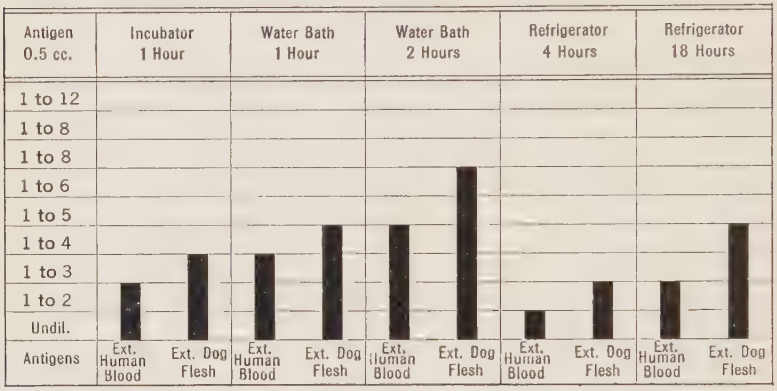
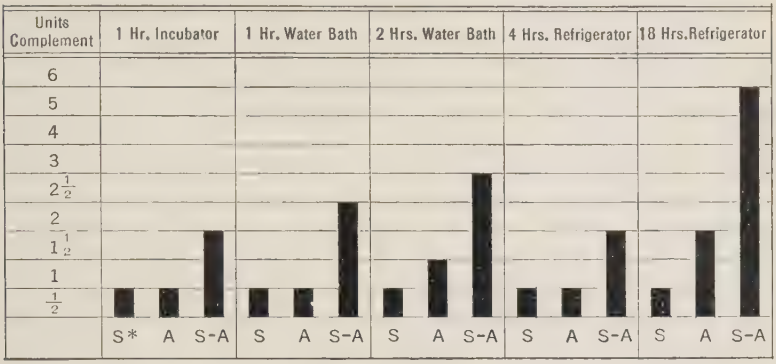


FIG. 24.—The influence of temperature and duration of primary incubation upon the anticomplementary activity of various extracts.

Water-bath incubation of one hour results in slightly greater non-specific fixation than occurs in an incubator for one hour; two hours in a water-bath generally results in the greatest degree of non-specific fixation. Four hours in a refrigerator at 6° to 8° C. generally results in less non-specific fixation than occurs in an incubator for one hour while eighteen hours in a refrigerator followed by ten minutes in a water-bath generally results in about the same degree of non-specific fixation as occurs in a water-bath for one hour. These experiments were conducted by using 2 units of complement with 0.5 cc. of varying dilutions of each antigen, to determine the smallest amount of antigen producing beginning inhibition of hemolysis in the different primary incubations of complement and antigen before the addition of hemolysin and corpuscles.

In additional experiments each antigen was employed in one-quarter its anticomplementary unit with 0.5 to 6 units of complement to determine the influence of kind of primary incubation upon the fixation of complement by antigen alone; the results of one of these titrations employing an antigen of human blood stain and anti-human serum is shown graphically in Fig. 25 as an example of the series. The results were quite similar to those stated above, namely, more complement is fixed during one hour in a water-bath than during one hour in an incubator. At 6° to 8° C. for four hours the degree of complement-fixation by these antigens was about the same or slightly less than occurred in an incubator for one hour;

after eighteen hours in a refrigerator plus ten minutes in a water-bath the degree of fixation was best of all.



* S = serum alone; A = antigen alone; S–A = serum and antigen.

FIG. 25.—The influence of kind and duration of primary incubation upon the non-specific and specific fixation of complement by antihuman serum and extract of dried human blood.

THE INFLUENCE OF KIND AND DURATION OF PRIMARY INCUBATION UPON THE SPECIFIC FIXATION OF COMPLEMENT BY EXTRACT ANTIGENS AND ANTISERA.

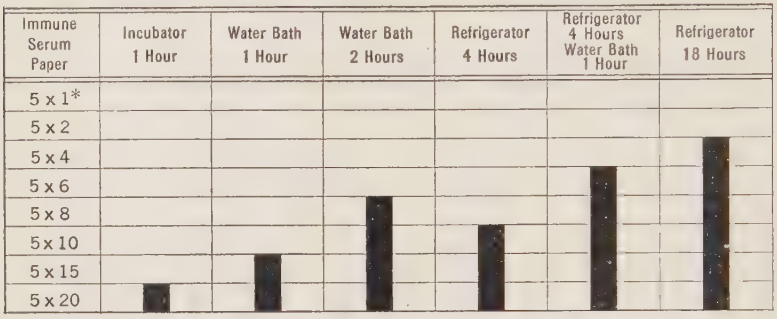
Experiments were conducted by using each antigen in an amount equivalent to one-quarter its anticomplementary unit with varying amounts of immune serum.

Rabbit and guinea-pig immune sera were employed and with the former due precautions were observed against non-specific complement-fixation. Some of these sera were preserved dried on filter papers, this method having been found very satisfactory for the preservation of immune sera for complement-fixation tests for the identification and differentiation of such soluble substances as blood stains, extracts of meats, etc.

Fig. 26 shows the results observed with an extract of human blood on cloth about four months and a rabbit antihuman serum dried on paper. The maximum degree of fixation occurred after a primary incubation of eighteen hours in a refrigerator followed by ten minutes in a water-bath; next in order were four hours refrigerator followed by one hour water-bath, two hours water-bath, four hours refrigerator, one hour water-bath and one hour incubator.

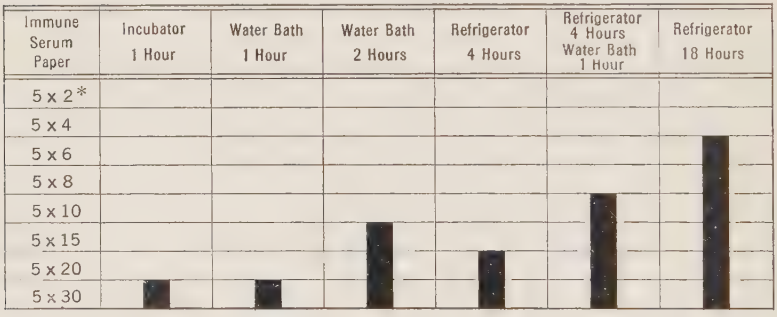
Fig. 27 shows the results observed with an antigen of cow's milk and a rabbit anticow lactoserum dried on paper. The results were similar to those given above, the maximum degree of complement-fixation occurring after a primary incubation of eighteen hours in a

refrigerator followed by ten minutes in a water-bath; the weakest reactions occurred with a primary incubation of one hour in an incubator.



* 5 x 1 mm., etc.

FIG. 26.—The influence of primary incubation upon complement-fixation with extract of *human blood stain.*



* 5 x 2 mm., etc.

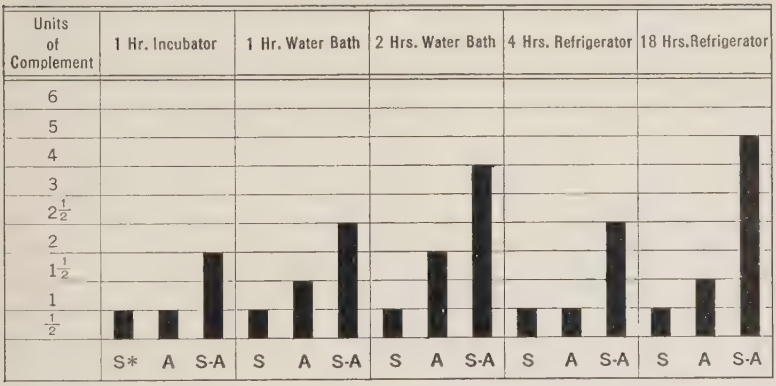
FIG. 27.—The influence of primary incubation upon complement-fixation with *cow's milk* and lactoserum.

Similar results were observed with the other antigens and antisera.

Duplicate experiments were conducted by using each antigen in an amount equivalent to one-quarter its anticomplementary unit with a fixed amount of immune serum and varying amounts of complement to determine the number of units of complement fixed by immune serum alone, antigen alone and by a mixture of serum and antigen in the different primary incubations. The results of these experiments were particularly clear and decisive and by subtraction the number of units of complement fixed by serun alone and antigen alone from the number fixed by the mixture of these, an accurate gauge of the degree of specific complement-fixation as influenced by the kind and duration of primary incubation was readily obtained.

Fig. 25 gives the results observed with an extract of dried human blood. With a primary incubation of one hour in an incubator, there occurred the specific fixation of 0.5 unit of complement; after one hour in a water-bath, 1 unit was fixed; after two hours in a water-bath, 1 unit was fixed; after four hours in a refrigerator, 0.5 unit was fixed; after eighteen hours in a refrigerator followed by ten minutes in a water-bath, 3 units were fixed. These values were obtained by subtracting from the number of units of complement fixed or absorbed by a mixture of serum and antigen, the total number fixed by serum alone and antigen alone.

Fig. 28 gives the results observed with an extract of uncooked beef and a rabbit antiox serum. After one hour in an incubator there occurred the specific fixation of 0.5 unit of complement (obtained by subtracting from the number of units fixed by a mixture of the serum and antigen the total number of units fixed by serum alone and antigen alone); after one hour in a water-bath, 0.5 unit was specifically fixed; after two hours in a water-bath, 1 unit; after four hours in a refrigerator, 1 unit; after eighteen hours in a refrigerator followed by ten minutes in a water-bath, 2.5 units of complement were fixed specifically.

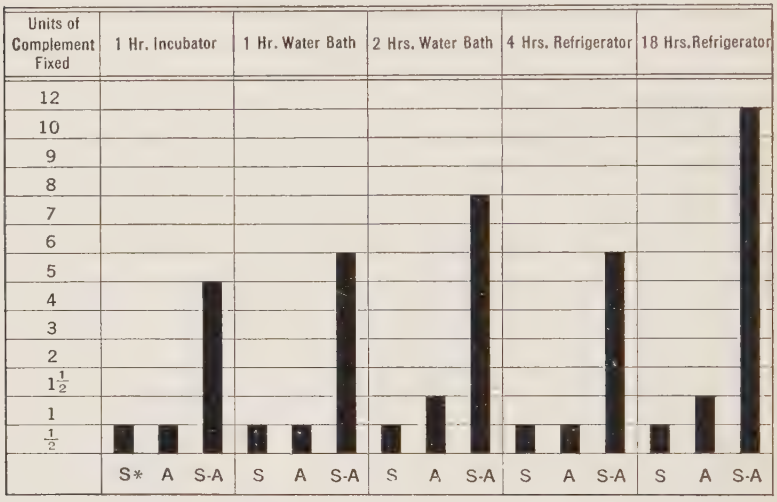


* S = serum alone; A = antigen alone; S–A = serum and antigen.

FIG. 28.—The influence of kind and duration of primary incubation upon the non-specific fixation of complement by antiox serum and an extract of beef flesh.

Fig. 29 gives the results observed with an antigen of cow's milk and a guinea-pig anticow lactoserum. After a primary incubation of one hour in an incubator there occurred the specific fixation of 3 units of complement; after one hour in a water-bath, 4 units; after two hours in a water-bath, 5.5 units; after four hours in a refrigerator, 4 units; after eighteen hours in a refrigerator followed by ten minutes in an incubator, 8.5 units of complement were fixed specifically.

Similar results were observed with other antigens and their immune sera, indicating that the least degree of specific complement-fixation by these antigens of soluble proteins and their immune sera, occurred during one hour in an incubator and the maximum degree after eighteen hours in a refrigerator followed by ten minutes in a water-bath.



S = serum alone; A = antigen alone; S–A = serum and antigen.

FIG. 29.—The influence of kind and duration of primary incubation upon the non-specific fixation of complement by cow lactoserum and antigen of cow's milk.

In complement-fixation tests for the identification and differentiation of proteins as blood stains, sera, extracts of meats and so forth, it is recommended therefore that the primary incubation of serum, antigen and complement be eighteen hours in a refrigerator at 6° to 10° C. followed by ten minutes in a water-bath, for the purpose of securing the maximum degree of specific complement-fixation.

CHAPTER XXVI.

SECONDARY INCUBATION AND THE TIME OF READING COMPLEMENT-FIXATION REACTIONS.

Following the primary incubation of serum, antigen and complement, the test corpuscles and hemolysin are added and the mixtures given the *secondary* incubation for the purpose of detecting whether any or all of the complement had been previously fixed or absorbed by antigen and antibody. The method of conducting this secondary incubation, however, has not attracted as much attention and study as it deserves. Probably the majority of serologists employ a water-bath or thermostat incubation of one hour at 38° C.

But several questions of some importance are involved. Is a water-bath superior to a thermostat? What is the optimum temperature? Should the secondary incubation be of a fixed period like one hour or is it better to remove the tubes and read the reactions as soon as the antigen and serum controls have undergone complete hemolysis? Should the reactions be read immediately or after the tubes have stood in a refrigerator for a few hours or over night for the partial or complete settling of corpuscles? I have attempted to answer some of these questions in a previous study[1] but a summary of the experiments and some newer data may be presented at this time.

PRINCIPLES OF SECONDARY INCUBATION.

Incubator versus water-bath.—Syphilitic and non-syphilitic sera were tested with an antisheep system by setting up two sets at the same time and under identical conditions with a primary incubation of eighteen hours at 8° C.; next day, hemolysin and corpuscles were added to the cold tubes of each set and secondary incubation conducted in an air incubator at 37° C. and in a water-bath at 37° C. for one hour. All tests were rendered quantitative by using each serum in graded amounts; the readings were made one hour after the completion of the secondary incubation. The results have been as follows:

1. Hemolysis in the water-bath is more rapid than in an air incubator; with the majority of sera the controls in the water-bath showed complete hemolysis in about one-quarter to one-half the time required in the air incubator. For this reason the water-bath is more convenient.

2. Secondary incubation in the air incubator, however, occasionally yielded more delicate reactions than observed in the water-bath; this is explained by the fact that the tubes warm up more slowly and permit the greater fixation of complement by syphilis antibody and tissue extract before a temperature is reached at which hemolysis is favored.

Temperature in Relation to Secondary Incubation.—To bring this fact out more clearly additional experiments were conducted with an antisheep system by setting up five sets with each of a series of syphilis and non-syphilitic sera, and after primary incubation of eighteen hours at 8° C. adding hemolysin and corpuscles to the cold tubes and conducting the secondary incubations in water-baths at 25°, 30°, 35°, 38° and 40° C. The results have been as follows:

1. The optimum temperature insofar as concerns rapidity of hemolysis in a water-bath is from 35° to 40° C.; hemolysis at 40° C. is somewhat more rapid than at 35° C.

2. With lower temperatures varying from 25° to 35° C. hemolysis is slower, but the reactions are more delicate due, probably, in part, to the fixation of more complement by syphilis antibody and tissue extracts at these lower temperatures before hemolysis takes place.

3. Secondary incubation at 25° C. is, however, too slow for practical purposes and with some sera results in incomplete hemolysis of the controls.

4. The optimum temperature for secondary incubation in a water-bath from the standpoints of sensitiveness and practicability is from 35° to 38° C. with an average of 37° C.

The Influence of Preliminary Warming of Tubes in a Water-bath Before the Addition of Hemolysin and Corpuscles.—As stated in Chapter XXIII if the tubes are placed in a water-bath at 37° C. for one hour after a primary incubation of eighteen hours at 8° C. before the addition of hemolysin and corpuscles, the reactions are rendered somewhat more sensitive. This result is due not only to a greater fixation of complement by syphilis antibody and tissue extract, but also to greater non-specific fixation of complement by extract alone and sera alone and to a greater destruction of complement; these non-specific factors outweigh the slight advantage of enhanced specific fixation and, therefore, the combined primary incubation of eighteen hours at 8° C. plus one hour at 38° C. could not be recommended.

However, with the technique of my *quantitative* test, a slight advantage is gained by warming the tubes in a water-bath at 37° C. for five to fifteen minutes, before the addition of hemolysin and corpuscles. As shown in Table 41 some sera show stronger reactions with the smaller amounts in tests conducted by giving the brief preliminary warming than in tests to which the hemolysin and corpuscles are added to the cold tubes. A preliminary warming of ten

minutes is almost as good as fifteen minutes; thirty minutes introduces more of the non-specific factors and for these reasons I believe that *the period of preliminary warming should be not less than ten or more than fifteen minutes.* Insofar as the percentage of positive and negative reactions is concerned, preliminary warming for fifteen minutes does not appear to exert any particular influence but even in the *qualitative* test employing 0.1 and 0.05 cc. amounts of serum, somewhat stronger reactions are frequently observed when the tubes are taken from the refrigerator after an incubation of sixteen to eighteen hours at 6° to 8° C. and placed in a water-bath at 38° C. for fifteen minutes before the addition of corpuscles and hemolysin.

TABLE 41.—INFLUENCE OF PRELIMINARY WARMING OF TUBES BEFORE THE ADDITION OF CORPUSCLES AND HEMOLYSIN UPON THE KOLMER QUANTITATIVE REACTION.

No.	Hemolysin and corpuscles added to cold tubes.						Tubes warmed at 38° C. for 15 minutes before addition of hemolysin and corpuscles.					
	0.1	0.05	0.025	0.005	0.0025	Control.	0.1	0.05	0.025	0.005	0.0025	Control.
1 . .	3*	1	—	—	—	—	3	2	1	—	—	—
12 . .	4	2	—	—	—	—	4	4	—	—	—	—
21 . .	2	—	—	—	—	—	2	1	—	—	—	—
45 . .	2	—	—	—	—	—	3	1	—	—	—	—
67 . .	4	1	—	—	—	—	4	3	1	—	—	—

* 4 = ++++; 3 = +++, etc.

A Fixed Period of Secondary Incubations versus a Variable Period for Each Serum.—The advantages of a fixed period for the secondary incubation is one of convenience when testing a large number of sera, inasmuch as inspection of serum controls and removal of the tubes when complete hemolysis has taken place, may not be a practical method by reason of the time and labor involved.

The advantage, however, of the variable period for individual sera is a check upon the possible influence of natural hemolysins when tests are being conducted with an antisheep or an antiox hemolytic system. Sera containing large amounts of natural hemolysin may yield somewhat weaker reactions with a fixed period of secondary incubation than with a variable period in which the tests are concluded and the reactions read as soon as the controls are completely hemolyzed. But even when natural antisheep hemolysin is removed routinely from all sera as in my method, the reading of reactions as soon as the antigen and serum controls are completely hemolyzed tends to give more sensitive readings than readings made

after a fixed incubation of one hour plus ten or fifteen minutes additional for further settling of corpuscles.

In one series of comparative tests, with 200 syphilitic and non-syphilitic sera Miss Yagle and I observed that readings made as soon as the controls were hemolyzed (about thirty-five minutes) agree with those made at the end of a fixed incubation of one hour in 93 per cent. In 3 per cent the preliminary readings gave true weakly positive reactions which were negative after the fixed period. In 2.5 per cent, however, the preliminary readings gave falsely positive reactions of weak degree due to slower hemolysis in the presence of antigen and serum; in 1.5 per cent the sera were anticomplementary in both. *But in 87 per cent of the positively reacting sera the readings were higher when made immediately after the hemolysis of the controls than at the end of one hour plus ten minutes or so at room temperature.*

Therefore, the advantage to be gained by the variable method of secondary incubation may be offset not so much by the greater time and labor involved as by the danger of reading falsely positive reactions. When the serum, antigen and hemolytic controls show just complete hemolysis there may be in the mixtures of serum and antigen, very slight inhibition of hemolysis which will disappear with a longer period of incubation. If the readings are made, however, when the controls just show complete hemolysis, these weak inhibitions of hemolysis in the main tubes may be erroneously interpreted as weakly positive reactions. This danger, however, may be removed if one waits *ten minutes after the complete hemolysis of the antigen control* before beginning the reading of reactions showing complete hemolysis of serum controls. With this precaution I believe that *experienced* serologists may adopt the variable method of secondary incubation as being somewhat more sensitive than the fixed method.

TIME OF READING REACTIONS.

Some serologists following Wassermann's original technique place the tubes in a refrigerator over night before the readings are made, which facilitates the readings and constitutes some advantage for the inexperienced.

In my opinion this practice inevitably leads to a small percentage of falsely negative reactions with weakly positive sera unless the balance of unfixed complement is rendered inactive by heating the tubes in a water-bath at 55° C. for ten minutes as recommended by me several years ago.[2]

Complement diluted 1 to 30 as in my test is always inactivated within ten minutes at 55° C. and this breaks up the hemolytic system so that placing the tubes in a refrigerator over night does not result in any further hemolysis which in the case of sera contain-

ing but small amounts of syphilis or other complement-fixing antibody may amount to a falsely negative result. Furthermore the corpuscles are not injured although slight hemolysis may be produced if the tubes are heated for fifteen to thirty minutes; ten minutes, however, is sufficient. Therefore, if it is preferred to place the tubes in a refrigerator over night for the settling of corpuscles to facilitate readings, I believe that it is advantageous to heat them at 55° C. for ten minutes immediately after the secondary incubation; this is true regardless of whether the natural hemolysins have or have not been previously absorbed but is especially important in case they have not been removed.

Fig. 30 shows in a graphic way the influence upon the sensitiveness of the syphilis reaction of the time adopted for the secondary incubation and the reading of results; the data are even more important and applicable to such bacterial tests as the gonococcus and tuberculosis complement-fixation reactions.

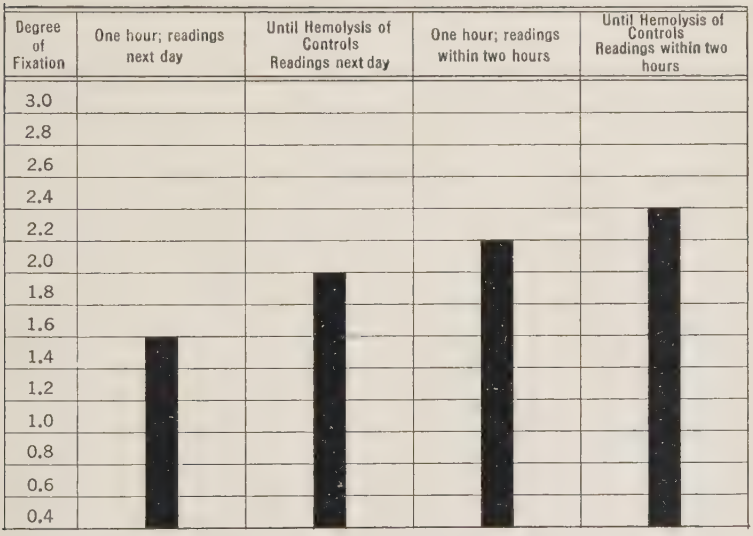


FIG. 30.—The influence upon the Kolmer complement-fixation test of the time allowed for the secondary incubation and the reading of reactions.

All the tests were conducted with syphilitic sera at the same time with a primary incubation of eighteen hours at 6° to 8° C. followed by fifteen minutes in a water-bath at 38° C. Each serum was tested by my quantitative method employing 5 doses of serum and the degree of fixation calculated by adding the plus signs and dividing by 5; for example a ++++, ++++, +++, −, − reaction was recorded as showing $\frac{11}{5}$ or a 2.2 degree of fixation. It is true that

with sera containing large amounts of antibody the differences are so slight as to be of little or no importance, but with sera containing small amounts of antibody and with either kind in case a quantitative method is employed, this part of complement-fixation technique is of considerable practical importance.

As shown by these experiments the *most sensitive reactions are those read ten minutes after complete hemolysis of the antigen control providing the serum controls are completely hemolyzed; this method can be recommended to experienced serologists when the number of sera being tested is not so large as to make the method impracticable or impossible. The next most sensitive reactions are those given a fixed secondary incubation of one hour and the readings made after the tubes have stood at room or refrigerator temperature for not more than two hours; this is probably the best routine method for testing large numbers of sera.* Reactions read after the tubes have stood in a refrigerator over night are less sensitive unless previously heated at 55° C. for ten minutes as described above.

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2. Kolmer: Am. Jour. Syph., 1921, **5**, 628.

CHAPTER XXVII.

READING SCALES AND METHODS OF RECORDING COMPLEMENT-FIXATION REACTIONS WITH SPECIAL REFERENCE TO THE KOLMER REACTION.

VARIOUS methods have been employed for reporting Wassermann and other complement-fixation reactions and considerable confusion has been thereby created in the minds of numerous physicians not especially experienced in this work. Of course the technique greatly influences the reactions and is the greatest source of variation in results reported by different laboratories with portions of the same serum or spinal fluid, but a uniform method of reporting reactions with the *same* method is highly desirable as emphasized by Kilduffe[1] and others.

METHODS FOR INTERPRETING AND RECORDING COMPLEMENT-FIXATION REACTIONS.

Complement-fixation reactions are generally recorded according to the degree of hemolysis or amount of non-hemolyzed corpuscles. This is usually judged by naked eye inspection of the tubes; Lyon and Eiman[2] have described a method of more accurate measurement of non-hemolyzed corpuscles by centrifuging in special tubes and Ivy[3] a method for measuring the degree of hemolysis by means of a colorimeter.

Two schemes for recording are widely employed; probably the best known is that credited to Citron in which the reactions are recorded somewhat as follows:

++++ 100 per cent inhibition of hemolysis or strongly positive.
+++ 75 per cent inhibition of hemolysis or moderately positive.
++ 50 per cent inhibition of hemolysis or weakly positive.
+ 25 per cent inhibition of hemolysis or very weakly positive.
± 10 per cent inhibition of hemolysis or doubtful.
− complete hemolysis or negative.

These graduations are only practical in "large volume" reactions, that is, in the original Wassermann reaction or such as use a sufficient number of corpuscles to show the different reactions with reasonable accuracy.

The second method is that employed by Craig and Vedder and

widely adopted for tests conducted with an antihuman hemolytic system:

++ 100 per cent inhibition of hemolysis.
+ 50 per cent inhibition of hemolysis.
× some inhibition of hemolysis but less than 50 per cent.
− complete hemolysis.

Boas prepares a hemoglobin scale to be used for recording the degree of hemolysis but his method is defective because it does not take into account such modifying factors as the antigen, complement and serum.

Bergeron and Normand[4] have overcome this objection by hemolyzing 10 different doses of corpuscles with hemolysin and complement and adding antigen and serum to each tube, giving a series of ten shades and permitting an accurate measurement of the degree of hemolysis.

Terry[5] had described a method of aiding naked eye readings and particularly differentiation among +++, ++ and + reactions by using tubes of uniform size (1 cm.) and reading at night holding the tube close to the eye, 18 to 24 inches from a 110 volt 25 to 40 Watt unfrosted Mazda bulb and looking for the filaments of the bulb. In a negative reaction the filaments are clearly seen; in a + they are also visible whereas in a ++ reaction they are invisible. For differentiation between a ++ and a +++ a specially constructed 3 mm. pipette is used in the same manner. The principle is good, but the technique requires too much time when a large number of quantitative tests are being conducted, the majority giving positive reactions.

AUTHOR'S METHOD FOR RECORDING COMPLEMENT-FIXATION REACTIONS.

My method employs 5 doses of serum and cerebrospinal fluid; the test is conducted with 0.5 cc. of a 2 per cent suspension of corpuscles in a total volume of 3 cc. These amounts easily permit the estimation of 100, 75, 50 and 25 per cent inhibitions of hemolysis corresponding to ++++, +++, ++ and +.

In my quantitative test employing 5 amounts of serum and spinal fluid the degree of positiveness or inhibition of hemolysis is recorded for each amount. With each dose of serum or spinal fluid the reaction may be read ++++, +++, ++, +, ×, or − giving a two-way quantitative test employing 5 dilutions with a scale of 6 possible readings on each, as per the following example with the serum of an untreated syphilitic in the secondary stage:

0.1 cc. serum = ++++
0.05 cc. serum = ++++
0.025 cc. serum = +++
0.005 cc. serum = +
0.0025 cc. serum = −

The above reaction may be recorded as 4431 — and reported as *very strongly positive.*

Sometimes the 0.1 cc. amount of serum yields a weaker reaction than the 0.05 cc. amount as per the following example of a reaction reported as *moderately positive:*

0.1 cc. +
0.05 cc. +++
0.025 cc. −
0.005 cc. −
0.0025 cc. −

In exceptional instances the 0.1 cc. amount may yield a falsely negative reaction as per the following example of a −2− − − reaction in a case of syphilis under treatment and reported as *moderately positive* because fixation occurred in the second tube:

0.1 cc. −
0.05 cc. ++
0.025 cc. −
0.005 cc. −
0.0025 cc. −

The cerebrospinal fluid of a case of paresis showed the following reactions, recorded as 44421 and reported as *very strongly positive:*

0.5 cc. fluid = ++++
0.25 cc. fluid = ++++
0.125 cc. fluid = ++++
0.0625 cc. fluid = ++
0.03125 cc. fluid = +

The strongest possible reactions with serum or cerebrospinal fluid would be recorded as 44444 and negative reactions as − − − − −; so far I have found but few sera and spinal fluids out of many thousands of tests, yielding 44444 reactions so that the range of doses appears satisfactory from the standpoint of a quantitative test.

Experience has shown that reactions with this technique may be interpreted as follows:

(*a*) *Very strongly positive* when positive reactions of *any degree* occur with the first 4 or all 5 dilutions.

(*b*) *Strongly positive* when the reactions are positive *in any degree* with the first 3 dilutions.

(*c*) *Moderately positive* when the reactions are positive *in any degree* with the first 2 dilutions.

(*d*) *Weakly positive* when the reactions are positive *in any degree* with the first or largest amount of serum or spinal fluid.

(*e*) *Doubtfuly positive* when the reactions are ± or show delayed hemolysis in the first tube.

(*f*) *Negative* when the reactions are negative throughout.

The following are examples of positive reactions observed in syphilis and especially illustrate the method of recording when the 0.1 cc. amount of serum gives a less sensitive reaction than the 0.05 cc. dose:

44443 = Very strongly positive
24431 = Very strongly positive
4431– = Very strongly positive
} fixation of *any degree* in first 4 or 5 tubes.

443– – = Strongly positive
231– – = Strongly positive
–21– – = Strongly positive
} fixation of *any degree* in any or all of the first 3 tubes.

44– – – = Moderately positive
34– – – = Moderately positive
–2– – – = Moderately positive
} fixation of *any degree* in the first 2 tubes.

3– – – – = Weakly positive
2– – – – = Weakly positive
1– – – – = Weakly positive
} fixation of *any degree* in the first tube.

Cerebrospinal fluid does not give these paradoxical reactions since the smaller doses do not give more sensitive reactions than the maximum amount of 0.5 cc.

READING SCALES.

With practice one may readily learn to read ++++, +++, ++, + and — reactions with the different doses of serum and spinal fluid and *a reading scale is not ordinarily required after experience has been gained*. Furthermore, it is almost impossible to devise any method which permits different workers to always agree on slight differences as between +++ and ++ or ++ and + reactions; nor does this appear to be of any particular importance. But serologists working in different laboratories read with slight differences and the use of a color scale may be advantageous from the standpoint of uniform readings.

Attempts to produce more or less permanent color scales with glycerin and gelatin have proved unsatisfactory; simple solutions of corpuscles in distilled water or after hemolysis by complement and hemolysin are likewise unsatisfactory because they do not show the modifications of color due to antigen, complement, etc.

A perfect reading scale should at least include the following:

1. The corpuscles of the day showing varying degrees of hemolysis.
2. The complement, because it is usually slightly tinged with hemoglobin and other pigments.
3. The antigen, which imparts an opalescence.
4. The patient's serum, because it is usually tinged with hemoglobin and other pigments.

The hemolysin is not necessary in an antisheep or antiox system because it is usually so highly diluted (1 to 500 or higher) as to be colorless; with the antihuman or antichicken hemolytic systems, the hemolysin may be included if used in fluid form, because they are generally employed in dilutions of about 1 to 50 or less.

With most of the reading scales so far proposed, only solutions of hemoglobin have been employed; experience has shown, however, *that reading the reactions according to the degree of hemolysis (color of supernatant fluid) and degree of inhibition of hemolysis (non-hemolyzed corpuscles) is actually easier and somewhat more accurate from the standpoint of uniformity of readings of the same reactions by different persons.*

Therefore, in the reading scale devised for my complement-fixation test (Chapter XXXI) both hemoglobin and corpuscles are included in addition to complement and antigen; the only ingredient omitted is serum because (1) some sera are apt to be just a little different in color from the majority and more especially because (2) each is used in five different amounts. Cerebrospinal fluid being practically colorless is likewise omitted.

The reading scale is composed of 5 tubes showing ++++, +++, ++, + and − reactions with the ingredients (corpuscles, antigen and complement) of each day's work. It is prepared at the close of the primary incubation and placed in the water-bath during the secondary incubation in order to equalize all conditions. The scale shows the proper degree of hemolysis and whole corpuscles and requires but a few minutes for its preparation. It has been found particularly useful for aiding naked eye readings of +, ++ and +++ reactions.

GRAPHIC FORMS.

Kilduffe[6] has devised a particularly useful form for recording and reporting complement-fixation reactions by my method which has been adopted by Rhamy and others. I have modified it slightly (Figs. 31 and 32) to show the kind of antigen employed in order to make it serviceable for reporting gonococcus and other complement-fixation reactions in addition to that for syphilis; also to use the −instead of the O signs for indicating a negative reaction.

Since the filing of a large number of these reports may add too much bulk to the history of a patient, I have devised the chart shown in Fig. 33 for use by the clinician which has proven quite satisfactory in my practice; it records the highest dilution of serum giving a positive reaction, which is of most importance, along with space for treatment records.

REPORT OF
COMPLEMENT FIXATION TEST

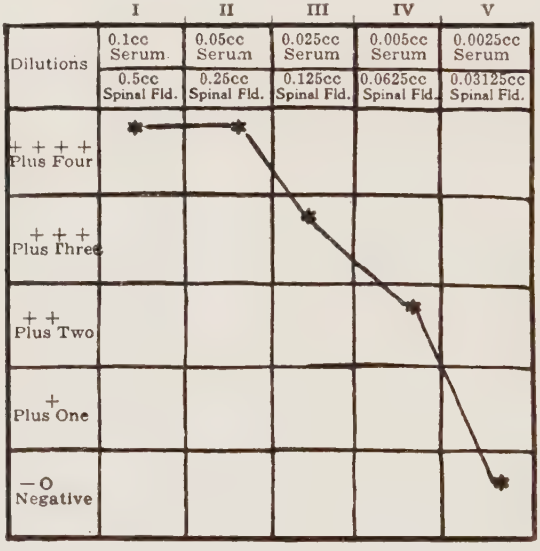
KOLMER QUANTITATIVE TECHNIC

Dr. ..... Date

Patient .....

Specimen of .....

Antigen Employed.....



Reading 4 4 3 2-0

Interpretation Very strongly positive

Fig. 31

REPORT OF COMPLEMENT FIXATION TEST

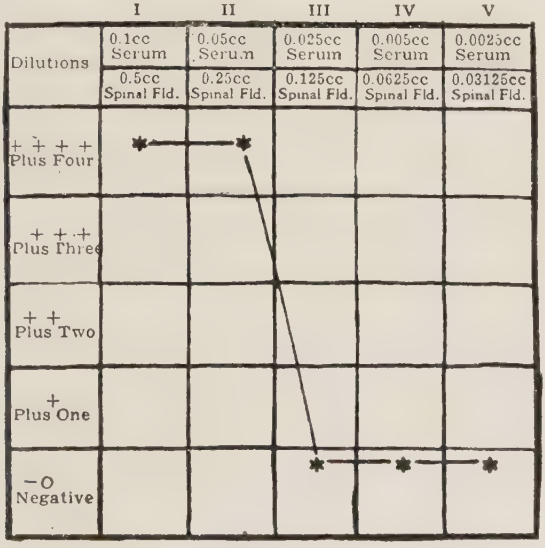
KOLMER QUANTITATIVE TECHNIC

Dr. ..... Date .....

Patient .....

Specimen of .....

Antigen Employed .....



Reading 4 4−0−0−0

Interpretation Moderately positive

Fig. 32

Since the cell count, protein, colloidal gold or mastic tests, etc. are usually conducted with spinal fluids in addition to the complement-fixation test I have employed the chart shown in Table 43 with uniform satisfaction for reporting results and filing with histories.

TABLE 42.—INFLUENCE OF NEO-ARSPHENAMIN UPON THE KOLMER COMPLEMENT-FIXATION REACTION IN ACTIVE TERTIARY SYPHILIS.

REACTIONS.	Serum (c.c.).	REACTIONS ACCORDING TO SMALLEST DOSE OF SERUM YIELDING A POSITIVE REACTION.											
		Mar. 5	Mar. 8	Mar. 22	Mar. 29	Apr. 5	Apr. 12	Apr. 19	Apr. 26	May 3	May 10	May 17	June 21
Very strongly positive	0.0025												
Very strongly positive	0.005												
Strongly positive	0.025												
Moderately positive	0.05												
Weakly positive	0.1												
Negative													
Treatment		Before	Neo. 0.9	Neo. 0.9	Neo. 0.9	Neo. 0.9	Neo. 0.9	Neo. 0.9	Neo. 0.9	Neo. 0.9	Neo. 0.9	Neo. 0.9	—

A more elaborate chart providing space for clinical notes, treatment, serological examinations of blood and spinal fluid as well as urinalyses, is shown in Tables 42 and 43.

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4. Bergeron and Normand: Presse Med., 1918, **26**, 472.
5. Terry: Proc. New York Path. Soc., 1917, p. 117.
6. Kilduffe: Am. Jour. Syph., 1925, **9**, No. 3; ibid., 1924, **8**, No. 3; Jour. Lab. and Clin. Med., 1923, **9**, No. 1.

TABLE 43.—CHART USED BY THE AUTHOR FOR RECORDING THE RESULTS OF CEREBROSPINAL FLUID EXAMINATIONS.

CEREBROSPINAL FLUID EXAMINATION

NAME: AGE: PHYSICIAN: DATE:
CLINICAL DIAGNOSIS:

PRESSURE (MILLIMETERS OF MERCURY)	AMOUNT REMOVED (C.C.)	PHYSICAL PROPERTIES	CELLS PER C. MM.	DIFFERENTIAL CELL COUNT			PROTEIN TESTS					COMPLEMENT-FIXATION REACTION	
							QUALITATIVE	QUANTITATIVE					
				LYMPH.	POLYS.	ENDOTHEL.	PANDY	0.2	0.1	0.05	0.025	BLOOD	SPINAL FLUID
Before													
After													

COLLOIDAL GOLD AND MASTIC REACTIONS*

REGISTRY NO.	COLOR REACTIONS	DILUTIONS OF SPINAL FLUID										REMARKS
		1 1:10	2 1:20	3 1:40	4 1:80	5 1:160	6 1:320	7 1:640	8 1:1280	9 1:2560	10 1:5120	
5	Colorless											
4	Pale Bule											
3	Blue											
2	Lilac or purple											
1	Red-blue											
0	Red-unchanged											

*____ gold curve; ..... mastic curve.

Smears:
Dextrose:
Quantitative Kolmer Reaction:
Serum 0.1 c.c.
Serum 0.05 c.c.
Serum 0.025 c.c.
Serum 0.005 c.c.
Serum 0.0025 c.c.

Bacteriological Examination:
Culture:

Animal Inoculation:
Quantitative Chlorids:
Quantitative Kolmer Reaction:
Spinal Fluid 0.5 c.c.
Spinal Fluid 0.25 c.c.
Spinal Fluid 0.125 c.c.
Spinal Fluid 0.0625 c.c.
Spinal Fluid 0.03125 c.c.

EXAMINED BY:

TABLE 44.—A CHART USED BY THE AUTHOR FOR RECORDING SEROLOGICAL EXAMINATIONS AND TREATMENT IN SYPHILIS.

Record of Complement-fixation Reactions.

Name: *Age:* *Address:*

Diagnosis:

Reactions.	Serum (cc.)	Dates of complement-fixation reactions.*											
Very strongly positive	0.0025												
Very strongly positive	0.005												
Strongly positive	0.025												
Moderately positive	0.05												
Weakly positive	0.1												
Negative	—												

* Reactions recorded according to smallest amounts of serum yielding positive reactions.

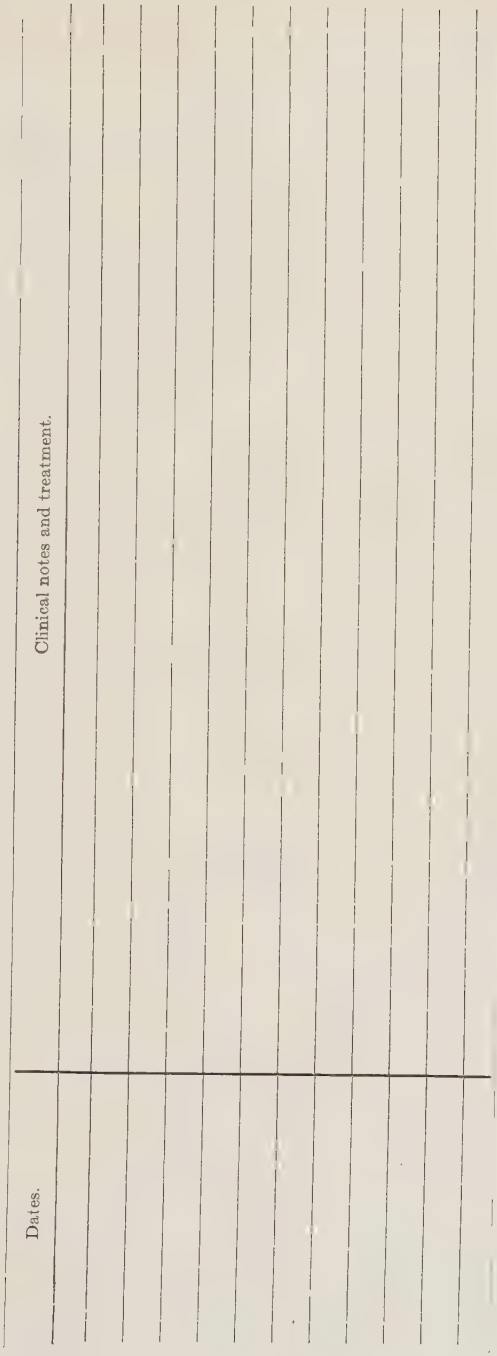
Dates.
Clinical notes and treatment.

TABLE 45.—REVERSE SIDE OF THE CHART SHOWN IN TABLE 44.

Records of Complement-fixation Reactions.

Degree.	0.1	0.05	0.025	0.005	0.0025	0.1	0.05	0.025	0.005	0.0025	0.1	0.05	0.025	0.005	0.0025	0.1	0.05	0.025	0.005	0.0025
Very strongly positive																				
Strongly positive																				
Moderately positive																				
Weakly positive																				
Negative																				
Date																				

Degree.	0.1	0.05	0.025	0.005	0.0025	0.1	0.05	0.025	0.005	0.0025	0.1	0.05	0.025	0.005	0.0025	0.1	0.05	0.025	0.005	0.0025
Very strongly positive																				
Strongly positive																				
Moderately positive																				
Weakly positive																				
Negative																				
Date																				

Cerebrospinal Fluid Examinations.

Date.	Pressure (mm. Hg.).	Amount cc.	Physical	Total cells, per c.cm.	Protein (globulin).	Sugar.	Colloidal gold reaction.	Complement-fixation reaction.				
								0.5	0.25	0.12	0.06	0.03

Urine Examinations.

Date												
Albumin												
Casts												

CHAPTER XXVIII.

NON-SPECIFIC COMPLEMENT-FIXATION BY THE NORMAL SERA OF RABBITS, DOGS AND MULES.

One of the most curious phenomena in complement-fixation is the property of normal rabbit, dog and mule sera of yielding non-specific reactions with the antigens employed in the Wassermann reaction; likewise and to an even greater extent with various bacterial antigens. Of further interest in relation to the mechanism involved is the fact that the same sera also yield positive Sachs-Georgi, Kahn and similar precipitation reactions with alcoholic tissue extracts.

This subject possesses a great deal of interest and practical importance not only from an academic standpoint in relation to its nature and mechanism, but more especially because rabbits are commonly employed in experimental syphilis and their sera used in Wassermann tests; also because immune rabbit sera are commonly employed in complement-fixation tests for the identification and differentiation of blood stains and other medico-legal work, for the differentiation of bacteria, etc. *Therefore unless complement-fixation technique is especially adapted for the sera of these animals it is easily possible to secure non-specific reactions and for this reason special methods for their testing have been described in Chapter XXXV.*

It is easily possible to avoid them by using more complement and more hemolysin and especially the former, than employed in tests with human sera and for this reason some observers have not encountered them at all because their technique happened to supply the excess of complement necessary for preventing their occurrence.

Review of Literature.—This property of normal rabbit serum for yielding non-specific complement-fixation reactions was first discovered in relation to the Wassermann test with alcoholic tissue extracts; it is now known, however, that even greater degrees of non-specific complement-fixation may occur with saline and watery extracts of tissues, bacteria and other substances. Michaelis[1] was probably first to observe the phenomenon since noted by many investigators, the literature being summarized in Table 46.

Emmanual[9] states that the reaction may be abolished by the administration of salvarsan and Epstein and Pribram[10] have made the same claim for bichloride of mercury but I have been unable to confirm their results.

Huddleson[15] has also observed positive reactions with normal sera but Kemp, Chesney and Poole[16] have observed only 0.3 per cent positive Wassermann reactions with normal rabbit sera because their method happened to supply a sufficient excess of complement to prevent them.

Rossi[17] has reported similar reactions with normal dog sera and Trist, Heist and myself[18] observed from 4 to 16 per cent positive Wassermann reactions with *unheated* sera in dose of 0.1 cc. and from 21 to 63 per cent with *heated* sera (55° C. for thirty minutes); with bacterial antigens, 54 to 70 per cent of *unheated sera* and 76 to 96 per cent of *heated sera* yielded positive reactions.

TABLE 46.—SUMMARY OF LITERATURE BEARING UPON THE FIXATION OF COMPLEMENT BY NORMAL RABBIT SERA.

Investigators.	Ref. No.	Number of rabbits tested.	Sera.	Antigens.	Positive reactions.*
Landsteiner, *et al.*	2	23	Heated	Extracts bacteria and tissue	43 per cent.
Fleischmann	3	...	....	Watery and alcoholic extracts of organs, lecithin, etc.	7 positive.
Blumenthal	4	...	Heated	Alcoholic extract of syphilitic liver	Positives.
Schilling and Hoesslin	5	5‡	Heated	Alcoholic extract of syphilitic liver	100 per cent.
Manteufel and Woithe	6	...	Heated	Watery and alcoholic extract of rabbit liver	Positive reactions.
Manwaring	7	...	Active	Various extracts and ferments	Positive reactions.
Browning and McKenzie	8	...	Heated	.....	Positive reactions.
Hellens	11	30	Active	Alcoholic extract of heart	27 per cent.
Hellens	11	30	Active	Alcoholic extract of liver	80 per cent.
Hellens	11	50	Heated	Alcoholic extract of heart	20 per cent.
Hellens	11	50	Heated	Alcoholic extract of liver	80 per cent.
Kolmer and Casselman	12	117	Heated	Alcoholic extract of syphilitic liver	13–20 per cent†
Kolmer and Casselman	12	117	Heated	Acetone extract of syphilitic liver	0–10 per cent†
Kolmer and Casselman	12	117	Heated	Acetone-insoluble lipoids	0–33 per cent†
Kolmer and Casselman	12	117	Heated	Cholesterolized alcoholic extract of heart	20–40 per cent†
Kolmer and Casselman	12	117	Heated	Saline extract of pallida cultures	None
Kolmer and Casselman	12	117	Heated	Alcoholic extract of pallida cultures	None
Kritchewsky	13	8	Active	Watery extract of gonococci, typhoid, etc.	20 per cent
Kritchewsky	13	27	Heated	Watery extract of gonococci, typhoid, etc.	64 per cent
Kolmer and Trist	14	75	Active	Alcoholic extract of tissues	5–15 per cent
Kolmer and Trist	14	75	Heated	Alcoholic extract of tissues	38–49 per cent
Kolmer and Trist	14	75	Active	Saline bacterial	31–42 per cent
Kolmer and Trist	14	75	Heated	Saline bacterial	51–62 per cent

* With 0.1 to 0.2 cc. serum.
† All reactions negative with 0.05 cc. serum.
‡ 55° C. for thirty minutes.

ANIMAL SERA YIELDING NON-SPECIFIC REACTIONS.

The normal sera of *rabbits* and *dogs* may yield therefore a high percentage of non-specific reactions with various antigens employed in the Wassermann and bacterial complement-fixation tests, the percentage and degree of fixation varying with the technique employed and with the state of the sera (heated or unheated). *Mule* sera react in practically the same manner.

Cattle sera may sometimes yield similar non-specific reactions with various antigens and especially bacterial extracts, when they

are tested after heating at 55° C. but not after heating at 62° C. for thirty minutes. *Sheep* sera behave in practically the same manner.

But *chicken* and *swine* sera have not yielded these reactions in the experiments of Rule and myself.

NATURE AND MECHANISM OF NON-SPECIFIC COMPLEMENT-FIXATION BY NORMAL RABBIT SERUM.

The cause of this phenomenon is unknown. Schilling and Hoesslin[5] originally believed that coccidiosis was responsible, but the investigations of Manwaring,[7] Browning and McKenzie,[8] Casselman and myself[12] and Trist and myself[14] have definitely disproven this assumption. I have shown[19] that both the serum lipoids and proteins are concerned in the process since sera extracted with ether and chloroform were less fixable while the enteral and parenteral administration of lipoids increased the antilytic and complement-fixing activity. As shown by Pearce and myself[20] chloroform and ether anesthesia may temporarily reduce or remove the property from the sera of dogs and rabbits.

The reactions are not due to the summation of the anticomplementary activities of sera and antigens; even perfectly fresh sera free of anticomplementary activity may produce the reactions.

Diet, sex and age do not appear to influence the phenomenon. *As a general rule the property of serum is constant for the animal throughout its life*, that is, if the serum reacts positively it continues to do so, although the degree or amount of complement-fixation is subject to fluctuations. Likewise *if the serum of an animal reacts negatively with a given antigen it will continue to do so with the same kind of antigen and same technique; with a different antigen or different technique, however, positive reactions may occur.*

Heating rabbit sera has considerable influence upon these non-specific reactions as shown by Trist and myself;[21] heating in the usual manner at 55° C. for one-half hour increases the tendency while at 62° C. for one-half hour the tendency is greatly reduced.

Precipitation Reactions With Normal Rabbit Serum.—Miss Yagle and I have observed that practically all rabbit sera (heated and unheated) yielding positive syphilis complement-fixation reactions in my method also yield positive Kahn precipitation reactions and I mention the fact here for the bearing it may have upon the nature and mechanism of the phenomenon and especially because it furnishes additional evidence of the fact that precipitation and complement-fixation in syphilis are probably due to the same substance in sera as discussed with more detail in Chapter IV.

Influence of Sachs' Method for Removing Anticomplementary Substances Upon Non-specific Complement-fixation.—As described in Chapter XXXVIII treating sera with $\frac{N}{30}$ hydrochloric acid accord-

ing to Sachs' method frequently removes anticomplementary substances but Richter, Rule and I have found that this method does not remove all of the substances in rabbit and dog sera yielding these non-specific complement-fixation reactions although it may reduce the degree of complement-fixation. I mention the results here not only because the method fails to remove this disturbing reaction for practical purposes but also because it indicates that the protein fractions of serum precipitated out in this method does not appear to carry the substance responsible for the reactions.

Mechanism of Non-specific Complement-fixation Reactions.—Indeed the mechanism is entirely unknown. Naturally one surmises that it may be due to the presence of normal or natural complement-fixing antibodies for the antigens employed, but the preliminary treatment of rabbit and dog sera with Kahn and various bacterial antigens for the purpose of absorbing these antibodies, has not removed more than a small fraction of them in the experiments of Miss Rule and myself. It may be that natural antibodies are responsible but the evidence is against this assumption; rather it would appear to be due to an obscure colloidal phenomenon involving the physical chemistry of the sera and profoundly influenced by heat.

THE KOLMER COMPLEMENT-FIXATION TEST IN RELATION TO NON-SPECIFIC REACTIONS WITH NORMAL RABBIT SERA.

Naturally the amount of complement or hemolysin or both employed in complement-fixation tests influences the occurrence and degree of these reactions; for these reasons the percentages of positive reactions reported by any investigator may not be corroborated by others employing a different complement-fixation technique. During the past five years I have restudied the subject with particular reference to my new technique in order to determine what modifications were required to remove the non-specific factor and render complement-fixation tests with the sera of rabbits, dogs and mules sufficiently sensitive and specific. The results are briefly summarized in the balance of this chapter while the details of technique are given in Chapter XXXV.

The main purpose was to investigate the problem with normal rabbit sera since the complement-fixation test has been found superior to the precipitin test for the identification and differentiation of the proteins of bacteria, blood, serum, milk and meats.

Technique Employed.—The technique employed was that of the new complement-fixation test described in Chapter XXXI except that a primary incubation of one hour in a water-bath at 38° C. was employed with all sera as well as the primary incubation of eighteen hours at 6° to 8° C. of this method. The essential features of the technical portion of this study were as follows:

1. The rabbits were healthy, unused and of adult size and on the usual diet of hay, oats and green vegetables.

2. The sera were tested unheated within twenty-four hours after collection of blood and after heating at 55° C. for fifteen and thirty minutes and at 62° C. for thirty minutes, to elicit the influence of heating sera upon the phenomenon.

3. The following antigens were titrated and used in doses equivalent to one-quarter the anticomplementary units; cholesterolized and plain alcoholic extracts of beef heart; acetone-insoluble lipoids of beef heart; heated saline suspension of typhoid bacilli and staphylococci; saline extracts of dog flesh and saline extracts of dried human blood. These extracts were selected as representatives of the usual extracts employed in the Wassermann and bacterial complement-fixation tests and tests for the identification of such soluble proteins as blood and extracts of meat.

4. The antisheep system was employed, the hemolysin being titrated with the complement of each day and the complement titrated in the presence of each extract with 2 units of hemolysin and employed in the complement-fixation tests in amounts of 2 full units with 2 units of hemolysin.

5. In order to elicit the influence of kind and duration of primary incubation each test was repeated with a primary incubation of one hour in a water-bath, as stated above.

6. In order to elicit the very important influence exerted by the amount of serum employed, each serum was tested in amounts varying from 0.1 cc. to 0.001 cc.

TABLE 47.—PERCENTAGE OF NORMAL RABBIT SERA IN AMOUNTS OF 0.1 TO 0.003 CC. YIELDING POSITIVE COMPLEMENT-FIXATION REACTIONS WITH VARIOUS ANTIGENS AND WITH WATER-BATH AND REFRIGERATOR PRIMARY INCUBATIONS.

Antigens.	Condition of sera.	Percentage giving positive reactions.	
		Water-bath.	Refrigerator.
Cholesterolized alcoholic extract	Unheated	42	83
Cholesterolized alcoholic extract	55° C. 15 min.	42	75
Cholesterolized alcoholic extract	55° C. 30 min.	50	92
Cholesterolized alcoholic extract	62° C. 30 min.	42	58
Plain alcoholic extract	Unheated	17	66
Plain alcoholic extract	55° C. 15 min.	17	66
Plain alcoholic extract	55° C. 30 min.	58	83
Plain alcoholic extract	62° C. 30 min.	17	66
Acetone-insoluble lipoids	Unheated	17	83
Acetone-insoluble lipoids	55° C. 15 min.	17	66
Acetone-insoluble lipoids	55° C. 30 min.	42	83
Acetone-insoluble lipoids	62° C. 30 min.	17	58
Saline bacterial	Unheated	37	75
Saline bacterial	55° C. 15 min.	33	58
Saline bacterial	55° C. 30 min.	42	71
Saline bacterial	62° C. 30 min.	42	58
Saline meat extract	Unheated	75	75
Saline meat extract	55° C. 15 min.	50	75
Saline meat extract	55° C. 30 min.	58	92
Saline meat extract	62° C. 30 min.	33	50
Saline dried blood extract	Unheated	33	58
Saline dried blood extract	55° C. 15 min.	33	58
Saline dried blood extract	55° C. 30 min.	42	75
Saline dried blood extract	62° C. 30 min.	25	25

The Influence of Heating Rabbit Serum Upon Non-specific Complement-fixation.—As shown in Tables 47 and 48 heating normal rabbit sera at 55° C. for fifteen minutes tends to reduce the tendency for positive non-specific reactions, that is, the percentage of sera yielding positive reactions in amounts of 0.1 to 0.001 cc. is usually reduced with all antigens. Heating the same sera at 55° C. for thirty minutes however, generally increases the tendency for non-specific reactions while heating at 62° C. for thirty minutes is followed by a decreased tendency; these facts were previously shown by Miss Trist and myself[14] and have been confirmed in this study.

TABLE 48.—SMALLEST AMOUNTS OF UNHEATED AND HEATED RABBIT SERA YIELDING POSITIVE REACTIONS WITH VARIOUS ANTIGENS IN THE NEW COMPLEMENT-FIXATION TEST.

Antigens.	Condition of sera.	Smallest fixing amounts.	
		Water-bath.	Refrigerator.
Cholesterolized alcoholic extract*	Unheated	0.003	0.003
Cholesterolized alcoholic extract*	55° C. 15 min.	0.013	0.003
Cholesterolized alcoholic extract*	55° C. 30 min.	0.013	0.025
Cholesterolized alcoholic extract*	62° C. 30 min.	0.025	0.025
Plain alcoholic extract . . .	Unheated	0.013	0.006
Plain alcoholic extract . . .	55° C. 15 min.	0.050	0.013
Plain alcoholic extract . . .	55° C. 30 min.	0.050	0.013
Plain alcoholic extract . . .	62° C. 30 min.	0.100	0.013
Acetone-insoluble lipoids . .	Unheated	0.006	0.006
Acetone-insoluble lipoids . .	55° C. 15 min.	0.025	0.025
Acetone-insoluble lipoids . .	55° C. 30 min.	0.025	0.013
Acetone-insoluble lipoids . .	62° C. 30 min.	0.100	0.013
Saline bacterial	Unheated	0.013	0.006
Saline bacterial	55° C. 15 min.	0.025	0.025
Saline bacterial	55° C. 30 min.	0.013	0.025
Saline bacterial	62° C. 30 min.	0.050	0.025
Saline meat extract	Unheated	0.006	0.003
Saline meat extract	55° C. 15 min.	0.013	0.025
Saline meat extract	55° C. 30 min.	0.013	0.025
Saline meat extract	62° C. 30 min.	0.050	0.025
Saline dried blood extract . .	Unheated	0.050	0.013
Saline dried blood extract . .	55° C. 15 min.	0.100	0.050
Saline dried blood extract . .	55° C. 30 min.	0.050	0.025
Saline dried blood extract . .	62° C. 30 min.	0.050	0.050

* With my new cholesterolized and lecithinized alcoholic extract of beef heart 0.025 cc. of normal rabbit serum (0.5 cc. of 1 to 50 dilution) rarely gives non-specific reactions.

Table 48 shows the smallest amounts of unheated and heated sera yielding positive reactions with the different antigens. It is to be remembered in this connection that not all rabbit sera yield positive reactions even in amounts as large as 0.1 cc. Fig. 33 shows the average smallest fixing amounts of sera from positive reacting rabbits with an alcoholic extract of heart and two bacterial

antigens in tests employing a primary incubation of eighteen hours at 8° C.

It shows at a glance the decreased tendency for positive reactions when sera are heated at 55° C. for fifteen minutes, followed by an increase when the sera heated at 55° C. for thirty minutes and by a marked decrease when heated at 62° C. for thirty minutes.

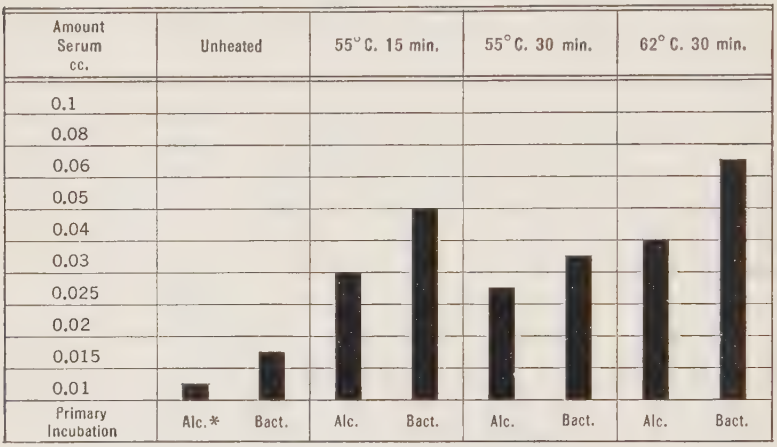

FIG. 33.—The influence of heating normal rabbit serum upon non-specific complement-fixation. Smallest average fixing amounts of serum with lipoidal and bacterial antigens.

* Alc. = plain alcoholic extract beef heart; bact. = average results with antigens of typhoid bacilli and staphylococci.

Primary incubation eighteen hours at 8° C.

The higher columns indicate more serum required and decreased tendency for non-specific fixation.

Percentage of Rabbit Sera Yielding Non-specific Reactions.—This varies according to the antigen, kind of primary incubation, condition of sera (heated or unheated), amount of serum employed in the tests and other technical factors. *With the technique employed in this investigation*, Table 47 expresses the percentages of sera yielding positive reactions with six different kinds of antigen and two primary incubations.

Of the alcoholic tissue extracts commonly employed in the Wassermann test, cholesterolized extracts yield the highest percentage of non-specific reactions.

A rabbit serum may react positively with an alcoholic tissue extract and negatively with a bacterial antigen or *vice versa; there is no one stock antigen that may be used for picking out positively reacting from negatively reacting rabbits. These preliminary tests should be done with the particular antigen or antigens to be employed in the study.*

Relation of Amount of Normal Rabbit Serum Employed to Non-specific Complement-fixation.—As previously stated, all rabbit sera used in this investigation were tested in amounts varying from 0.1 to 0.001 cc.; *it is worthy of special note that when unheated sera are employed negative reactions may occur with 0.1 and 0.05 cc. whereas smaller amounts may yield positive reactions.* Examples of these reactions were observed with all the antigens in this study except the cholesterolized extract, which was the most sensitive and yielded more positive reactions than the other lipoidal extracts.

A number of examples of these reactions with different antigens are presented in Table 49 and are ascribed to the influence of natural hemolytic complement and thermolabile antisheep hemolysin when unheated sera are tested in amounts of 0.05 and 0.1 cc. Of these two modifying factors the influence of native complement is probably the more important, inasmuch as the major portion of natural antisheep hemolysins in rabbit sera are of the thermostabile variety and exert almost as much influence in tests employing heated sera as in those employing fresh unheated sera.

TABLE 49.—EXAMPLES OF *Falsely Negative Reactions* WITH THE 0.1 CC. AMOUNTS OF NORMAL RABBIT SERA.

Sera.	Antigen.	Primary incubation.	Amounts of serum.				
			0.1	0.05	0.025	0.013	0.006
Unheated	Alcoholic extract of heart	38° C. 1 hr.	−*	3	3	1	−
Unheated	Alcoholic extract of heart	38° C. 1 hr.	−	1	4	4	1
Unheated	Acetone-insoluble lipoids	6° C. 18 hrs.	−	1	3	1	−
Unheated	Dog muscle	38° C. 1 hr.	−	1	2	1	−
Unheated	Dog muscle	38° C 1 hr.	−	1	3	−	−
Unheated	Dog muscle	6° C. 18 hrs.	−	−	1	2	1
Unheated	Dog muscle	6° C. 18 hrs.	−	1	3	2	−
Unheated	Staphylococci	38° C. 1 hr.	−	1	2	1	−
Unheated	Staphylococci	6° C. 18 hrs.	−	2	4	4	2
Unheated	Staphylococci	6° C. 18 hrs.	−	1	3	2	−
Unheated	Staphylococci	6° C. 18 hrs.	−	1	3	4	1
Unheated	B. typhosus	6° C. 18 hrs.	−	4	4	1	−
Unheated	B. typhosus	6° C. 18 hrs.	−	2	4	4	2
Unheated	B. typhosus	6° C. 18 hrs.	−	2	3	−	−
Unheated	Extract dried blood	6° C. 18 hrs.	−	−	1	4	2

* − = negative; 1 = +; 2 = ++; 3 = +++; 4 = ++++.

The particular practical significance of these observations is that when unheated rabbit sera are tested for non-specific complement-fixation prior to immunization of the animals, it is unsafe to rely upon the results observed with amounts larger than 0.05 cc.; with heated sera these larger amounts of serum may be safely employed.

Table 48 shows the *smallest* amounts of normal rabbit sera (unheated and heated) yielding positive reactions with six different antigens and with two kinds of primary incubation.

With the new complement-fixation technique employed in this investigation including a primary incubation of eighteen hours at

6 to 8° C., it would appear that the following are the maximum amounts of rabbit serum permissible to avoid non-specific reactions with different antigens in case preliminary tests are not done:

0.003 cc. with ordinary cholesterolized extracts but 0.025 cc. (0.5 cc. of 1 to 50) with my new extract.

0.006 cc. with plain extracts and acetone-insoluble lipoids.

0.005 cc. with bacterial antigens and meat extracts (0.5 cc. of 1 to 100 dilutions).

Therefore *a rabbit immune serum employed with this complement-fixation method for the Wassermann test, identification and differentiation of bacteria and differentiation of proteins should be sufficiently powerful to yield positive reactions with 0.5 cc. of 1 to 50 or higher dilutions in order to avoid the element of non-specificity.* Of course with selected rabbits yielding negative reactions in preliminary tests larger amounts of immune sera may be employed.

Relation of Primary Incubation to Non-specific Complement-fixation by Normal Rabbit Sera.—A primary incubation of eighteen hours at 6° to 8° C. greatly increases the tendency for positive reactions as compared with water-bath incubation of one hour. As shown in Fig. 34 slight fixation may occur with some sera (not all) immediately after mixing serum, antigen and complement but longer incubations increase the degree of complement-fixation, the curves of this non-specific fixation of complement being influenced by primary incubation in the same manner as specific complement-fixation in bacterial and syphilis reactions.

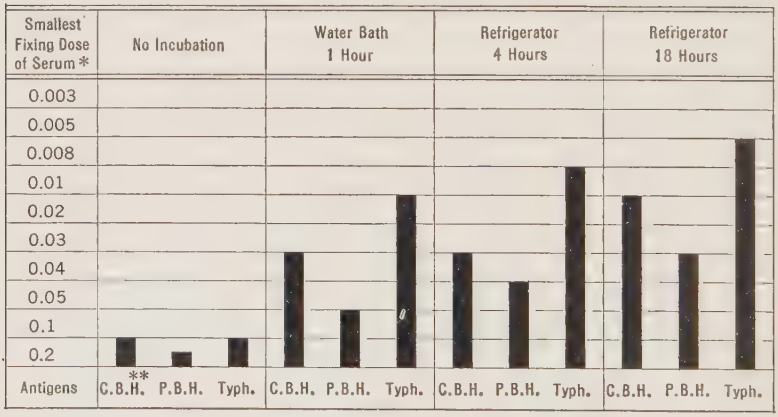

FIG. 34.—The influence of kind and duration of primary incubation upon non-specific complement-fixation by normal rabbit sera (averages obtained with the sera of six positively reacting rabbits.)

* Sera heated at 55° C. for fifteen minutes.

** C. B. H. = cholesterolized alcoholic extract of beef heart; P. B. H. = plain alcoholic extract of beef heart; Typh. = a saline suspension of B. typhosus. All used in one-quarter anticomplementary units.

Since the sera of some rabbits yielding particularly strong reactions may fix complement immediately after the mixture of serum, complement and antigen *the question arises whether or not the use of low titered immune rabbit serum hemolysins may not produce slight inhibitions of hemolysis in complement-fixation tests by means of this non-specific action of rabbit serum.* Experiments have shown that when normal human serum, antigen and complement are incubated for one hour in a water-bath followed by the addition of 0.1 or 0.05 cc. normal rabbit serum, 2 units of antisheep hemolysin and sheep corpuscles, that the rabbit serum may fix some of the complement before hemolysis takes place. *Not infrequently the dose of 2 units of rabbit antihuman hemolysin serum is 1 cc. of 1 to 10 or 2 cc. of 1 to 20 dilution and it is possible that such amounts of serum from some rabbits may be sufficient for producing slight fixation of complement in complement-fixation tests when there is free antigen and free complement at the end of the primary incubation, which is the case with negative sera.*

The Influence of the Amounts of Complement and Hemolysin Employed Upon Non-specific Complement-fixation by Normal Rabbit Serum.—As previously stated it is readily understood that the technique exerts a tremendous influence upon the occurrence and degree of non-specific complement-fixation by normal rabbit sera; this is especially true in relation to the amount of complement employed as shown by Trist and myself,[14] and accounts for the variable results reported by different investigators (Table 46). This fact is worthy of emphasis and percentages and doses are to be interpreted only in relation to the technique of complement-fixation employed; those reported in this chapter may not represent what would be observed in a similar study employing a different technique.

As previously stated, 2 full units of complement with 2 units of hemolysin were employed in this study. The influence of more complement in reducing the tendency for non-specific fixation with different antigens is shown in Table 50 in tests employing a primary incubation of eighteen hours at 6° to 8° C.; in duplicate tests employing water-bath incubation of one hour the influence of complement was even more marked.

TABLE 50.—THE INFLUENCE OF AMOUNT OF COMPLEMENT EMPLOYED UPON NON-SPECIFIC COMPLEMENT-FIXATION REACTIONS WITH NORMAL RABBIT SERUM.

Antigens.	Reactions with increasing amounts of complement.						
	1 unit.	2 units.	3 units.	4 units.	5 units.	6 units.	8 units.
Cholesterolized alcoholic extract	+	+	+	−	−	−	−
Plain alcoholic extract	+	+	−	−	−	−	−
Bacterial suspension	+	+	+	+	+	−	−

Duplicate tests employing 2 full units of complement but 3, 4, 5, 6, 8, 10 and 12 units of hemolysin have yielded somewhat similar results, that is, 3 or more units of hemolysin reduces the percentage and degree of non-specific complement-fixation.

As described in Chapter XXXV, I employ therefore a modification of my test in which 4 full units of complement are used instead of 2; under these conditions sera may be used in amounts as high as 0.1 cc. after heating at 55° C. for fifteen minutes. If the regular technique is employed with 2 full units of complement and 2 of hemolysin the largest dose of serum (heated at 62° C. for thirty minutes) should not exceed 0.025 cc. (0.5 cc. of 1 to 50 dilution) with syphilis antigens or 0.0125 cc. (0.5 cc. of 1 to 40 dilution) with bacterial antigens; both methods are given.

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CHAPTER XXIX.

PRINCIPLES OF THE COMPLEMENT-FIXATION REACTION FOR THE IDENTIFICATION AND DIFFERENTIATION OF SOLUBLE PROTEINS LIKE BLOOD AND SEMINAL STAINS, SERA, MEATS AND MILK ADULTERATIONS.

In my experience the complement-fixation reaction has proven much more satisfactory than the precipitin reaction for the identification and differentiation of soluble proteins and especially of blood stains. As in the latter the matter of proper quantitative relations between antigen extract of blood or other substance like serum, semen, meat, etc., and homologous immune serum has required careful study but with these worked out satisfactorily, the complement-fixation test has proven more sensitive and conclusive than the precipitin test. These fundamental principles are summarized in the present chapter while the actual technique is given in Chapters XXXVI and XXXVII and a discussion of their specificity and practical application in Chapters L, LI and LII.

PRINCIPLES INVOLVED.

In the syphilis and bacterial complement-fixation tests the antigens are known and the antibodies sought for in the serum or other fluid being tested. In these reactions, however, the conditions are reversed since the nature of the antiserum is known and that of the antigen unknown in the extract of material being tested. Therefore to determine if a blood stain is of human origin an extract of it is tested with a known antihuman blood serum and under proper technical conditions a positive reaction is acceptable as evidence of its human origin although the test cannot determine whether the blood is from a particular human being. In other words, the complement-fixation reaction, like the precipitin reaction, may be used to identify blood or serum according to species but neither is capable of differentiating among individuals belonging to the same species. Likewise the complement-fixation reaction can detect the presence of dog or cat flesh in sausages but cannot identify either as coming from a particular dog or cat.

THE ANTISERUM.

An antiserum must be prepared for each antigen sought for in the extract of blood, meat, etc. For example, in medico-legal work a blood stain may be of human, cat, dog or other origin. As a

general rule the chief or sole interest of the courts is in whether or not a stain or other material is of human blood and in this case the material need be tested only with a known antihuman serum. But if negative reactions are observed the extract would require retesting with other antisera like antidog, antichicken, antiox, anticat, etc., for identification; it would be useless to use antisera of animals known not to be available like those of the gorilla and other of the higher apes. In testing sausages for adulteration it is usually sufficient to use antidog, anticat and other immune sera for the flesh of the lower animals suspected of being present.

These antisera are prepared by the immunization of rabbits and titrated for their content of complement-fixing antibody. *To be satisfactory they should give ++++ reactions with homologous antigens in a dose of at least 0.5 cc. of 1 to 200 dilution* since each serum is used in a dose of 2 or more units and should not exceed 0.5 cc. of 1 to 100 dilution in order to avoid the non-specific reactions sometimes yielded by normal rabbit serum discussed in the preceding chapter. As a general rule more potent sera are readily prepared and this is advisable since it is much better to use a serum in a dose of 0.5 cc. of 1 to 500 containing at least 2 or more fixing units than to use 0.5 cc. of a 1 to 100 dilution.

Each immune serum is heated at 62° C. for thirty minutes before use because this still further reduces the chances of error due to non-specific complement-fixation. As shown in Table 51 there is some loss in antibody but not enough to ruin acceptable sera.

TABLE 51.—THE INFLUENCE OF HEATING IMMUNE SERA UPON COMPLEMENT-FIXATION TESTS FOR THE IDENTIFICATION OF BLOOD STAINS AND MEATS.

Antigens.†	Immune sera.	Sera heated 56° C. 15 minutes.				Sera heated 62° C. 30 minutes.			
		0.006 cc.	0.003 cc.	0.0015 cc.	0.0008 cc.	0.006 cc.	0.003 cc.	0.0015 cc.	0.0008 cc.
Ext. dried human blood	Antihuman	++++	+++	+	−	++++	+	−	−
Ext. dried sheep blood	Antisheep No. 1*	++	++	+	−	++	+	−	−
Ext. dried sheep blood	Antisheep No. 2*	++++	++++	++++	+++	++++	++++	++++	+
Ext. dried dog blood	Antidog No. 1	++++	++++	++++	+	++++	++++	++	+
Ext. dried dog blood	Antidog No. 2	++++	++++	+++	−	++++	+++	+	−
Ext. dog meat	Antidog No. 1	++++	++++	++++	+++	++++	++++	++	+
Ext. dog meat	Antidog No. 2	++++	++++	++	−	++++	+++	−	−

* Antisheep hemolysin removed.
† All antigens employed in one-quarter anticomplementary units.

If rabbits are injected with sheep blood or sheep muscle extracts there is produced antisheep hemolysin in addition to antisheep complement-fixing antibody. If tested with an antisheep hemolytic system this would introduce the error of an excess of hemolysin as shown in Table 52. This could be avoided by using an antiox or antihuman hemolytic system but is easily corrected by absorbing

the antisheep hemolysin by treating each cubic centimeter of the immune serum with 0.1 cc. of washed sheep corpuscles and removing the sensitized corpuscles by centrifugation one hour after standing in a refrigerator.

TABLE 52.—ANTISHEEP HEMOLYSIN SHOULD BE ABSORBED FROM ANTISHEEP SERUM EMPLOYED IN COMPLEMENT-FIXATION TESTS FOR THE DETECTION OF SHEEP BLOOD OR FLESH.

	Antisheep serum plain.*					Antisheep serum hemolysin free.*				
Dried blood antigens.†	0.006	0.003	0.0015	0.00075	Serum control.	0.006	0.003	0.0015	0.00075	Serum control
Sheep	−	−	+	++	−	++++	+++	++	++	−
Human	−	−	−	−	−	−	−	−	−	−
Dog	−	−	−	−	−	−	−	−	−	−

* Serum heated at 62° C. for thirty minutes.
† Each antigen used in one-third anticomplementary unit.

THE EXTRACTS.

The extracts of material to be identified, as of blood on fabrics, walls, wood and grass, etc., of semen on garments, of meats in sausages adulterated with dog or cat flesh, etc., must be prepared in such manner as to contain sufficient amounts of the soluble proteins. But even finely divided suspensions may be employed. As a general rule the stock extract should contain at least 1 part of substance to 100 parts of diluent and be neutral in reaction. It should contain enough protein to give a reaction in the heat and acetic acid or nitric acid tests as applied to urine for the detection of albumin.

Each extract must be titrated for its anticomplementary activity in order to avoid using any in a dose alone capable of interfering with hemolysis.

Optimum Dosage of Extract and Antiserum.—Prezone reactions are particularly apt to occur in these complement-fixation reactions. Therefore it is a serious mistake to believe that the most sensitive reactions always occur with the largest amounts of extract and antiserum.

As shown in Table 53 *the best results are observed by using the extract antigen in variable amounts with a constant dose of antiserum.* When conditions are reversed and the extract is used in a constant dose with variable amounts of immune serum (Table 54) the reactions are not nearly as sensitive although prezone reactions are not so likely to occur. In both tables the same extract and antisera were used in tests conducted at the same time and with the same hemolytic system.

TABLE 53.—COMPLEMENT-FIXATION TESTS EMPLOYING VARIABLE AMOUNTS OF ANTIGEN WITH FIXED AMOUNTS OF ANTISERA.

Antisera in dose of 0.5 cc. of 1:100.	Antigens.	Dilutions of antigens in dose of 0.5 cc.								
		1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600
Antihuman	Human serum	4*	4	4	4	4	4	4	3	1
Anticow	Cow serum	2	4	4	4	4	4	4	4	3
Antisheep	Sheep serum	2	4	4	4	4	4	3	2	2
Antihorse	Horse serum	3	4	4	4	4	4	4	3	2
Antihog	Hog serum	1	4	4	3	2	1	—	—	—
Antichicken	Chicken serum	1	4	4	2	—	—	—	—	—
Antidog	Dog serum	4	4	4	4	4	4	4	3	2

* 4 = ++++; 3 = +++; 2 = ++; 1 = +; — = complete hemolysis.

TABLE 54.—COMPLEMENT-FIXATION TESTS EMPLOYING FIXED AMOUNTS OF ANTIGEN WITH VARIABLE AMOUNTS OF ANTISERA.

Antigens 0.5 of 1:100.	Antisera.	Dilutions of antisera in dose of 0.5 cc.						
		1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
Human serum	Antihuman	4	4	3	—	—	—	—
Cow serum	Anticow	4	4	3	—	—	—	—
Sheep serum	Antisheep	4	3	2	—	—	—	—
Horse serum	Antihorse	4	4	1	—	—	—	—
Hog serum	Antihog	3	—	—	—	—	—	—
Chicken serum	Antichicken	4	1	—	—	—	—	—
Dog serum	Antidog	4	4	1	—	—	—	—

Either method may be employed as shown in Tables 55 and 56 but I believe it better to use the antiserum in a fixed dose with varying amounts of extract antigen which is just the reverse of the usual procedure in syphilis and bacterial complement-fixation reactions, in which the antigen is constant and the serum variable.

While these tables show the results observed with extracts of blood and serum, identical results were observed in similar tests employing meat, milk and seminal extract antigens with their homologous antisera.

TABLE 55.—THE COMPLEMENT-FIXATION TEST FOR THE FORENSIC DIFFERENTIATION OF DRIED BLOOD STAINS USING VARYING AMOUNTS OF EXTRACTS OF STAINS WITH CONSTANT AMOUNTS OF IMMUNE SERA.

Blood stain.	Antihuman serum 0.002 cc.					Antisheep serum 0.003 cc.				
	Ext. 0.2	Ext. 0.1	Ext. 0.05	Ext. 0.025	Ext. 0.0125	Ext. 0.2	Ext. 0.1	Ext. 0.05	Ext. 0.025	Ext. 0.0125
Human	++++	++++	++	+	—	—	—	—	—	—
Human	+++	+	—	—	—	—	—	—	—	—
Human	++++	++	+	—	—	—	—	—	—	—
Sheep	—	—	—	—	—	++++	++	+	—	—
Sheep	—	—	—	—	—	++	—	—	—	—
Sheep	—	—	—	—	—	++++	++	—	—	—

TABLE 56.—THE COMPLEMENT-FIXATION TEST FOR THE FORENSIC DIFFERENTIATION OF DRIED BLOOD STAINS IN DOSE OF 0.5 CC. OF 1 TO 100 AND VARYING AMOUNTS OF RABBIT IMMUNE SERA.

Rabbit.	Serum.	With extract dried human blood.				With extract dried sheep blood.				With extract dried beef blood.			
		Serum 0.002	Serum 0.001	Serum 0.0005	Serum control.	Serum 0.002	Serum 0.001	Serum 0.0005	Serum control.	Serum 0.002	Serum 0.001	Serum 0.0005	Serum control.
1	Antihuman	++++	+++	++	−	−	−	−	−	−	−	−	−
2	Antihuman	++++	++++	+++	−	−	−	−	−	−	−	−	−
3	Antihuman	+++	++	−	−	−	−	−	−	−	−	−	−
4	Antihuman	++++	++++	++	−	−	−	−	−	−	−	−	−
5	Antihuman	++++	++++	++	−	−	−	−	−	−	−	−	−
6	Antisheep*	−	−	−	−	+++	+	−	−	−	−	−	−
7	Antisheep	−	−	−	−	++++	+++	+	−	−	−	−	−
8	Antiox	−	−	−	−	−	−	−	−	+++	+	−	−
9	Antiox	−	−	−	−	−	−	−	−	++	+	−	−
10	Antiox	−	−	−	−	−	−	−	−	++++	+++	+	−

* Antisheep hemolysin removed from sera.

PART III.

TECHNIQUE OF COMPLEMENT-FIXATION METHODS.

CHAPTER XXX.

STANDARDIZATION OF COMPLEMENT-FIXATION TECHNIQUE WITH SPECIAL REFERENCE TO SYPHILIS AND THE KOLMER REACTIONS.

Probably no laboratory test has been subject to as much favorable and unfavorable criticism and to as many modifications in technique as Wassermann's serum test for syphilis. Owing to the fact that the reaction is not strictly or biologically specific for syphilis and requires several biological reagents of varying properties, it is readily subject to error in both a positive and negative way unless carefully and intelligently understood and conducted, as is true of the Kahn and numerous other precipitation tests for this disease.

Many of the proposed modifications of the original technique have been demanded by an increasing experience and knowledge of the reaction and several have undoubtedly served to improve its delicacy and value, but the employment of different methods by various persons had led to wide variation in the results, confusion in regard to the actual value of the test and insistent demand for a standard method.

DISCREPANCIES IN THE WASSERMANN REACTION.

Within recent years several publications have drawn particular attention to discrepancies in the results of Wassermann reactions with the same serum in different laboratories and indeed, to varying results reported by the same serologist on specimens of blood from the same individual withdrawn at the same time and under identical conditions. For example, Uhle and MacKinney[1] who submitted specimens of blood from each of 292 individuals to from four to ten laboratories in 1915, found that "there is 1 chance in 5 that the tests will agree;" Phelps[2] in a similar analysis of the results of Wassermann tests with 358 specimens of blood divided and sent to from

two to four laboratories, found that the results agreed in but from 52 to 65 per cent and Wolbarst[3] in a previous study of 37 cases by two or more serologists, found that the results agreed in 70 per cent and disagreed either slightly or absolutely in 30 per cent. More recently Palmer[4] has reported upon the variable results on specimens sent to eight different laboratories and as an example of the influence of technique upon the results of complement-fixation and precipitation tests in syphilis in 1926 I may cite here those observed by Dr. Ruth Gilbert and Miss Virginia Langworthy of the New York State Department of Laboratories in a study conducted under the auspices of the American Public Health Association.

The sera of 252 individuals selected by Dr. Parounagian of the Bellevue Clinic in New York City and Dr. H. W. Potter of the State Institution for the Feeble Minded were sent to six different laboratories; they included treated and untreated cases of syphilis in different stages and healthy non-syphilitic controls.

Of the cases of treated, untreated and suspected cases of syphilis the *percentage of positive reactions* varied according to the methods and antigens employed as follows:

	Per cent.
Post-Graduate Hospital of New York City	28.7 to 66.7
Illinois State Department of Health	29.7 to 30.7
Western Reserve University	27.0 to 31.4
Massachusetts Department of Health	13.5
Graduate School of Medicine of the University of Pennsylvania (Kolmer)	50.2
New York State Department of Health	39.3 to 62.0

Kahn tests were conducted by three of these laboratories with the following *percentages of positive reactions* and showed a similar variation in results despite the fact of its alleged greater simplicity of technique:

	Per cent.
Illinois State Department of Health	31.7
Western Reserve University	51.2
Michigan State Department of Health (Kahn)	43.6

I believe all serologists of experience agree that a certain percentage of discrepancies are to be expected with the use of different hemolytic systems and reagents and particularly different antigens, even granting that all tests were conducted with the requisite technical care and attention to details. I do not know the legal value of the Wassermann reaction, but I surmise that the testimony of several serologists under close cross examination upon the witness stand would bid fair to outshine the exhibition of alienists in regard to conflicting statements. But the Wassermann reaction is only one of numerous laboratory tests requiring standardization to increase value and reliability; for example, it is well known that

the much simpler Widal, Kahn, Sachs-Georgi and other reactions may yield varying results in different laboratories with portions of the same specimen of blood and indeed, conflicting reports on the results of urinalysis with portions of the same specimen of urine containing traces of albumin or sugar, may be expected. Nevertheless, it is the overwhelming consensus of opinion that the Wassermann reaction possesses great value in the diagnosis of syphilis and as a serological guide to treatment, owing largely to the fact that the individual physician has learned to rely upon the work of at least one of his colleagues; but, owing to the nature and importance of the disease to the individual and community, standardization of the technique for the serum diagnosis of syphilis is especially desirable in order to increase the delicacy and reliability of the reaction.

THE DEMAND FOR STANDARDIZATION OF TECHNIQUE.

The demand for this standardization of the Wassermann reaction has come from both clinicians and serologists who have observed marked discrepancies in the results reported by different laboratories testing portions of the same serum. I need mention here but few of the many pleas published from time to time like those of Shropshire and Watterson,[5] Litterer and Watterson,[6] Stillians,[7] Ray,[8] Langston[9] and Kilduffe.[10] In Germany[11] considerable advance has been made in this direction since a special permit is required for those conducting the test in commercial laboratories and who are likewise required to use antigens which have been officially standardized.

Under these conditions physicians not particularly informed in this work may well ask why confidence should be placed in the Wassermann reaction. As a matter of fact however, physicians themselves are largely to blame for past and present conditions because of a failure to exercise due care in the selection of those serologists or laboratories to whom so important a test is entrusted. Altogether too many entertain the erroneous idea that a Wassermann test is simply a Wassermann test irrespective of by whom it is done. For the majority of clinicians this would appear to be a logical situation but it is very illogical to those who understand the test and know the many opportunities for error due to variation in the qualities of the biological reagents employed, not to mention the element of the personal equation in relation to exactness in the technique.

In other words, there is no one way for conducting the Wassermann test. The original method is lacking in sensitiveness and has demanded improvement. To meet this requirement numerous modifications have been adopted, so that not a few experienced serologists have made changes here and there in the technique

which the clinician should realize and understand may greatly modify the sensitiveness and practical specificity of the reaction. As I pointed out in a former communication[12] the majority of these modifications are still known by most clinicians as the 'Wassermann reaction," but the phrase has really come to be only a synonym for complement-fixation in syphilis, frequently signifying something very different from its original meaning.

Such being the case, it is apparent that the mass of data accumulating on the value of the complement-fixation test in different stages of syphilis can be only approximately correct, and that much of it is worthless and misleading. Furthermore, it is apparent that accurate and acceptable data bearing on the influence of specific treatment on the reaction and the relative merits of different systems of treatment are not possible under existing conditions. Dr. A., using the method of treatment advocated by Dr. B. is liable to observe serological results different from Dr. B's, owing largely to the fact that he or his serologist is employing a different method for conducting the Wassermann reactions and especially a different antigen in differing amounts. *All experienced serologists know the tremendous influence exerted on the Wassermann reaction by this one reagent, not to mention numerous other sources of variation;* for these reasons identical reactions with portions of the same sera tested in different laboratories could not be expected, and if reported would probably be received by experienced serologists with much skepticism. The same is true of the Kahn, Sachs-Georgi and other precipitation tests.

THE ADVANTAGES AND DISADVANTAGES OF STANDARDIZATION.

If serologists could reach an agreement on a standardized technique of superlative merit, it would solve the present difficulties and gradually strengthen or regain the confidence of the profession in a reaction that is basically sound and of proved merit. At least four distinct advantages would be gained: (1) A wider use of the test for the diagnosis of syphilis, which could not be otherwise than helpful for the diagnosis, control and treatment of this disease; (2) the accumulation of data of greater scientific value bearing on the practical value of the test in the different stages of syphilis; (3) a general improvement in the value of the reaction as a serological guide to therapy, and (4) a means for comparing different methods of treatment according to their influence on the reaction, which would tend to aid in the crystallization of our knowledge in this field.

A possible disadvantage of adoption of a standardized test would be the stifling of further investigation; however, so much is as yet unknown regarding the mechanism of the reaction and there are so

many different ways of doing the same thing, that this is scarcely to be expected as a result, so far at least, as concerns those serologists who are capable of conducting meritorious investigations in this field. I doubt whether any serologist or group of serologists would have the temerity to advocate a certain technique as a "cure all" or as constituting the final word in the subject. The advantages mentioned above could probably be gained, and still leave and demand continued investigation and further improvements. In my opinion, the adoption of a standardized technique would probably actually stimulate investigation and, by focusing attention on a certain technique, bring out its merits and demerits to the general improvement of the complement-fixation test, not only for syphilis but also for other infections as well.

SOURCES OF ERROR IN THE COMPLEMENT-FIXATION REACTION.

As is well known there are many sources of error in the technique of the Wassermann and other complement-fixation reactions as is true of agglutination, precipitation and other reactions. These sources of error may be (*a*) *unavoidable or biological* and (*b*) *avoidable or technical*. By *unavoidable* sources of error I refer to that change occurring in the sera of persons suffering with diseases other than syphilis, as frambesia, which are said to be responsible for positive Wassermann reactions in the absence of syphilis under rigidly correct technical conditions. In frambesia the reason is readily surmised inasmuch as the etiological agent is a spirochete bearing a very close resemblance to *Spirochæta pallida* and, like the latter, highly vulnerable to the spirocheticidal action of arsenobenzol. A review of the literature on the Wassermann reaction and particularly the earlier papers, shows that positive reactions have been reported by some one at some time in practically all diseases to which human flesh is heir, but we now know that many of these reports must have been due to *avoidable* errors in technique. By these I refer to those present in the various biological reagents of the test as in complement, hemolysin and particularly the antigen; likewise to errors due to careless technique. A standardized method aims to detect and avoid all avoidable errors although it cannot account for the personal equation of the individual serologist in regard to exactness in work, beyond making the test and the method of reading the results as simple, definite and clear as possible.

THE AUTHOR'S INTERPRETATION OF STANDARDIZATION.

Possibly the easiest way of standardizing the Wassermann test would be to adopt the original technique with a change in the antigen. But such a proposal would receive little recognition, not only because the original technique is defective in sensitiveness but also

because the antigen is the greatest single source of contention and disagreement. *A valuable and practical step in advance would be immediately gained by the adoption of a standard antigen for the syphilis reaction prepared, titrated and distributed by central laboratories.* Possibly the preparation and standardization of the hemolysin would be likewise advisable.

In my opinion "standardization" means a real, earnest and unbiased study of different methods and of each and every phase of the complement-fixation test, for the purpose of determining by actual trial what is best and incorporating the facts into a test which will have for its purposes the establishment of a technique: (1) of superlative sensitiveness; (2) of practical specificity; (3) of technical accuracy and uniformity of results; (4) one yielding a true quantitative reaction; (5) one as simple; and (6) as economical as possible.

My colleagues and I have endeavored to approach the problem in this manner, the results of our studies upon the fundamentals being given in preceding chapters and the actual details of technique being given in the following chapters, an attempt being made to fulfil these requirements by the following procedures:

Meeting the Requirement of Sensitiveness.—(1) By using highly sensitive antigen; (2) using relatively large amounts of antigen; (3) using relatively large amounts of serum and spinal fluid; (4) removing natural antisheep hemolysin from all sera; (5) heating sera for only fifteen minutes at 55° C.; (6) using a mixture of guinea-pig serum complements prepared in a manner tending to increase sensitiveness to fixation; (7) mixing serum and antigen for a brief period before the addition of complement; (8) using a primary incubation of from fifteen to eighteen hours at from 6° to 8° C. followed by ten to fifteen minutes in a water-bath at 38° C.; (9) by close adjustment of the hemolytic system to cold primary incubation; (10) by using an antisheep or antiox hemolytic system; and (11) by reading the reactions immediately after the conclusion of the secondary incubation.

Meeting the Requirement of Practical Specificity.—(1) By using antigens of superior antigenic sensitiveness and possessing the minimum of anticomplementary action; (2) by adjustment of the hemolytic system to cold primary incubation; (3) by adjustment of the dose of antigen to cold primary incubation; and (4) by using numerous controls.

Meeting the Requirements of Technical Accuracy and Uniformity in Results. (1) By adoption of the principle that pipetting relatively large amounts of fluid (from 0.3 to 1 cc.) tends to greater accuracy than measuring smaller amounts (less than 0.2 cc.); (2) by using a total volume of 3 cc., with sufficient corpuscles and test-tubes of suitable size to yield clear, sharp and easily read reactions; and (3)

by using a reading scale furnishing hemoglobin in solution and non-hemolyzed corpuscles in proper portions.

In regard to uniformity in results, it must be emphasized that the anticomplementary activity of serum or spinal fluid is very important in relation to reactions. For this reason, tests conducted with portions of the same blood in different cities or even in the same city, cannot be expected to yield absolutely similar results if serologists vary in their methods of preserving blood until the tests are conducted. Serologists working in the same or different laboratories with portions of the same blood should agree on the question of positive or negative reactions. Slight variations in the degree of positiveness may occur when serologists are working in different laboratories, but these do no harm as long as the primary question of whether a serum does or does not yield a positive or negative result is solved, and particularly with serums yielding borderline weakly positive or doubtfully negative reactions.

Meeting the Requirement of a Quantitative Reaction.—This has been accomplished by employing a series of dilutions or doses of serum or spinal fluid. Extensive trials have shown that 5 doses (6 tubes, including the serum control) are sufficient and a method has been worked out whereby these may be prepared rapidly and accurately.

By using only the first and second doses of serum or spinal fluid, the test becomes a qualitative one, like other methods in present use, should any one wish to employ a shorter technique.

Meeting the Requirement of Economy.—This refers to both time and materials. My technique is not a short cut method as I am convinced that the principles involved in complement-fixation are too intricate, the reagents too subject to variation and our knowledge of the mechanism of the reaction too meager to permit the evolvement of a short cut and simple method fulfilling the requirements of a standard test. Doubtless the time and labor involved for conducting the new test will prevent its adoption by some serologists, and especially those who feel obliged to render a report on the same day that a specimen is received, for which regrettable state of affairs physicians are largely responsible; but I have endeavored to adhere to the principle that accuracy should never be sacrificed for speed and labor saving. The new technique provides for a quantitative and a qualitative test; I use the former routinely because it requires but little more time.

Insofar as materials are concerned both the quantitative and qualitative tests easily fulfil the requirement of economy since each require but 0.3 cc. serum and 1.5 cc. of spinal fluid. In the new quantitative test 1 cc. of guinea-pig serum is usually sufficient for examining 6 to 7 sera or spinal fluids; in the qualitative test this amount suffices for at least 12 sera or spinal fluids including all

controls. In the original Wassermann test, which is a qualitative test only, 1 cc. of complement is sufficient for testing 8 sera or spinal fluids including the usual controls. Complement is the most expensive reagent and the new test easily meets the requirements of economy in this and all other materials. The amounts of blood corpuscles and hemolysin required are so small as not to be worthy of discussion.

Meeting the Requirement of Simplicity.—Simplicity is only a relative term, as the simplest technique is a complicated problem for the inexperienced and insufficiently trained worker, whereas a more complicated technique is perfectly simple for the experienced serologist.

Certainly this and no other test should be entrusted to insufficiently trained and inexperienced workers, be they graduates of medicine or laboratory technicians. As a general rule, an apprenticeship of at least four months on full time should be served under a well-trained serologist in a well-conducted, busy and hard-working laboratory devoted to the principle that nothing may be sacrificed for accuracy and especially mere speed in returning reports. Each specimen should be carefully labelled and given a serial number to avoid the very regrettable error of confusion of specimens and it is my custom to enjoin *silence* and orderliness of the laboratory upon all workers as the best work cannot be done in the midst of levity, confusion, noise and dirt.

Furthermore the best work is only possible in those laboratories conducting a large number of tests twice or more times weekly. It is almost impossible to conduct tests under the best of conditions when only a few specimens are examined weekly or at longer intervals and the same is true of the precipitation tests. It is much better for physicians with private laboratories attached to their offices and small institutions to pool this work under a skilful and experienced serologist because it would be likely to be done under much more reliable and satisfactory conditions and likewise with less expense which, in the case of syphilitics requiring numerous tests under treatment, is a consideration of importance to the majority.

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CHAPTER XXXI.

TECHNIQUE OF THE KOLMER COMPLEMENT FIXATION TESTS FOR SYPHILIS.

The methods described with detail in this chapter are those based upon the studies summarized in Chapters VIII to XXVII and are exactly the same as described by me in 1922[1] except the natural antisheep hemolysin is routinely removed from all sera. The antisheep hemolytic system is described but the antiox system may be employed in exactly the same manner in those localities where sheep blood cannot be obtained. I hope that those who are learning complement-fixation technique will be careful to read and study the preliminary chapters because every step in the following methods is based upon investigation. And I particularly hope that all who adopt my methods will conduct them *exactly* as described. To use my antigen in another method, as frequently done, does not by any means conduct my test since the antigen was built up especially for the particular hemolytic system and primary incubation described.

EQUIPMENT.

Test-tubes.—These should have rounded bottoms, no lips and measure 85 by 15 mm. outside.* It is important that the diameter of the tubes vary not more than $\frac{3}{4}$ mm. either way if the color scale for reading reactions is employed.

Flasks and Cylinders.—For mixing large volumes, as in the preparation of corpuscle suspension or dilutions of complement serum, ordinary graduated cylinders may be used, if carefully selected on the basis of accuracy in graduations. For measuring any amount of fluid under 50 cc. it is better to use an accurate 10 cc. pipette and reserve the graduated cylinders or flasks for measuring larger volumes.

Pipettes.†—There should be a separate 1 cc. pipette for each serum or spinal fluid to be tested; 5 and 10 cc. pipettes are also required. For placing 1.2 cc. of saline solution in the first tube of each set-up, I have had prepared a special pipette carrying 14.4 cc. sufficient for 12 tubes at one filling. All are illustrated in Fig. 35.

Automatic Pipettes—These are extremely useful and save a great deal of time and labor especially in pipetting saline solution, antigen,

* Arthur H. Thomas Co. Catalogue No. 9446.
† Arthur H. Thomas Co. Catalogue No. 8174.

complement, corpuscles and hemolysin. In my test the dose of antigen is always contained in 0.5 cc. of dilution, the complement in 1 cc., the corpuscles in 0.5 cc. and the hemolysin in 0.5 cc.; automatic pipettes deliver these amounts with great rapidity and accuracy. I have used the one invented by Dr. John L. Laird* and shown

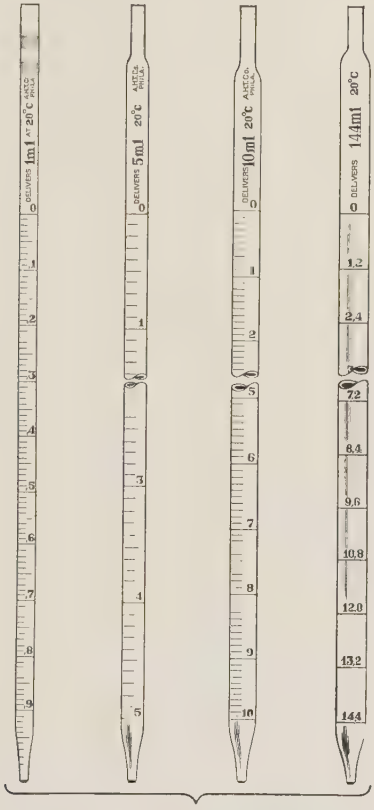

FIG. 35.—Author's serological pipettes.

in Fig. 36 and that by Dr. Frank W. Hartman, shown in Fig. 37† with success and satisfaction; that invented by Dr. W. F. Lorenz[3] is also highly recommended.‡ When 50 or more tests are to be done at one time I unhestitatingly recommend the use of one of these machines.

* Marketed by Edward P. Dolbey Company of Philadelphia.

† Marketed by the Detroit Laboratory and Hospital Supply Co., 8019 Third Avenue, Detroit, Mich.

‡ Marketed by J. S. Hipple, University of Wisconsin, Madison, Wis.

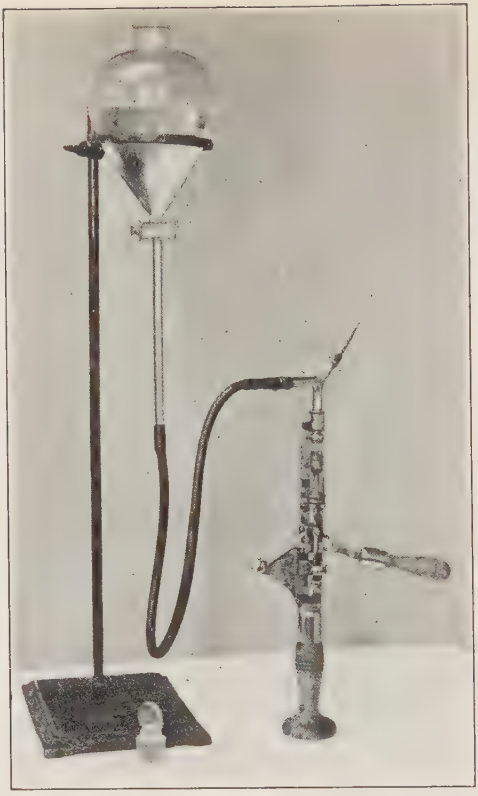

FIG. 36.—The Laird automatic pipette.

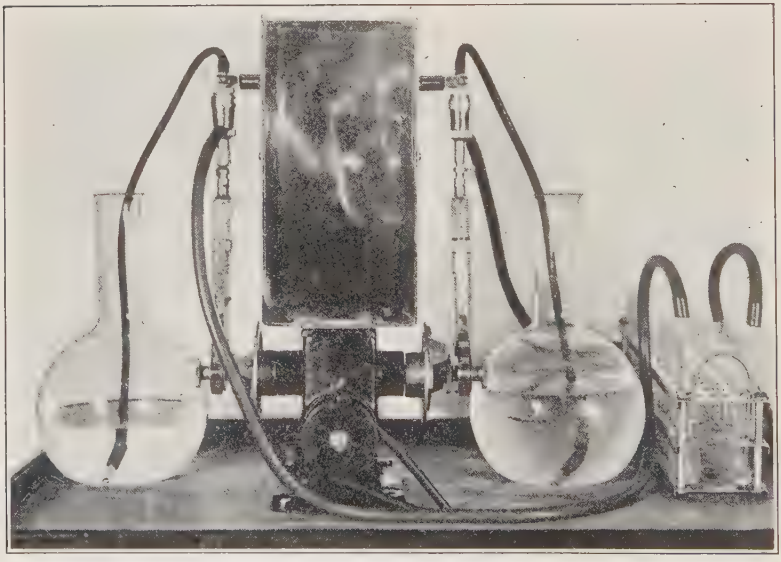

FIG. 37.—The Hartman automatic pipette.

Centrifuge Tubes.—In preparing 2 per cent suspensions of sheep corpuscles the volume of cells is read off after the last washing and for making accurate readings I devised the special centrifuge tubes shown in Fig. 38; they are sufficiently accurate and have been employed during the past nine years with very satisfactory results.*

FIG. 38.—Special centrifuge tubes divised by the author for the washing of corpuscles.

FIG. 39.—Wire rack used by the author for complement-fixation tests; carries 72 tubes. (From Kolmer: Infection, Immunity and Biologic Therapy, W. B. Saunders Co., Publishers.)

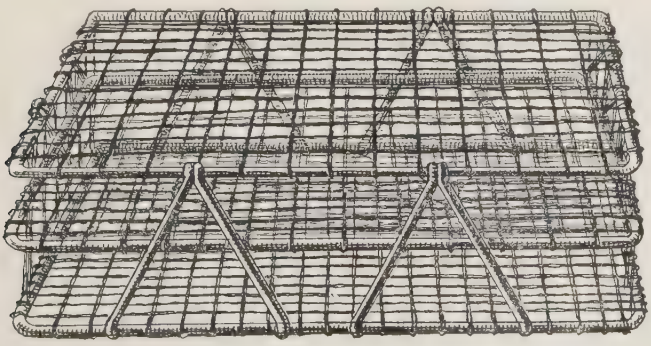

FIG. 40.—A larger wire rack used by the author for complement-fixation tests; carries 144 tubes. (From Kolmer: Infection, Immunity and Biologic Therapy, W. B. Saunders Co., Publishers.)

* Arthur H. Thomas Co. Catalogue Nos. 3118 and 3119.

Test-tube Racks.—The galvanized rack shown in Fig. 39 carries 12 rows of 6 tubes each;* that shown in Fig. 40 carries 24 rows of 6 each.† The former is sufficient for 12 quantitative or 24 qualitative tests; the latter carries 24 quantitative or 48 qualitative tests.

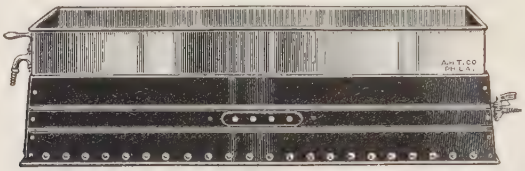

FIG. 41.—A large water-bath used by the author for secondary incubation in complement-fixation tests.

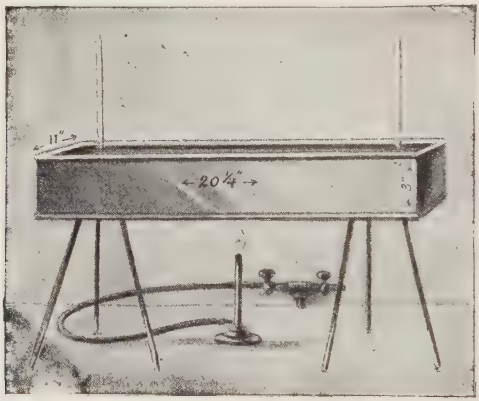

FIG. 42.—A very simple and efficient water-bath used by the author for the inactivation of sera and for secondary incubation. (From Kolmer: Infection, Immunity and Biologic Therapy, W. B. Saunders Co., Publishers.)

FIG. 43.—An electric water-bath for the inactivation of sera and secondary incubation.

Water-baths.—A simple and inexpensive water-bath for heating sera at 55° C. and conducting the secondary incubation at 38° C. is shown in Fig. 41;‡ a much simpler pan made by any tinsmith is shown in Fig. 42. When carrying water to the depth of 8 to 10 cm.

* Arthur H. Thomas Co. Catalogue No. 1726.

† No. 1727.

‡ Arthur H. Thomas Co. Catalogue No. 1722 (small model); No. 1723A (large model).

the temperature of either pan may be maintained at 55° C. or 38° C. with very little care and attention. That shown in Fig. 43 is for electrical heating and the one which I had the privilege of using maintained temperatures of 55° C. and 37° to 38° C. in a very satisfactory manner.*

Refrigerator.—The primary incubation is fifteen to eighteen hours at 6° to 10° C. All of my earlier work and tests were conducted in a satisfactory manner with an ordinary ice refrigerator. When kept well filled with ice and the doors not opened too frequently, the lower compartments gave the correct temperature. During the past seven years I have used electric refrigerators with great success and satisfaction since they may be nicely adjusted and as made at present give little or no trouble.

PREPARATION OF GLASSWARE.

1. As stated in Chapter VIII new and used glassware should be *chemically clean* and preferably (but not necessarily) sterile. New and used *test-tubes* should be washed inside and out in hot soapy water, thoroughly rinsed in running water (a final rinsing in distilled water is not ordinarily required), placed in galvanized wire or copper baskets upside down and heated in a hot-air oven until a piece of cotton placed in the oven has turned a light brown color. It is unnecessary to plug them with cotton.

In some laboratories it is customary to boil the tubes in soapy water, rinse in tap water, immerse briefly in 1 per cent hydrochloric acid to neutralize the alkali of the soap, rinse thoroughly and sterilize. But the contents of used tubes are not infectious (as those used in bacteriological work may be) and in my experience boiling and immersion in acid are unnecessary as routine procedures, although it is occasionally necessary to do this with tubes which are spotty and cloudy; *but the rinsing in running water after immersion in the acid bath must be particularly thorough.*

While it is preferable to prepare the glassware on the day preceding the tests, the tubes are fit for use any time within a period of several days after preparation.

2. New and used *flasks and cylinders* may be prepared in the same manner. It is advisable to plug them with cotton if glass stoppers are not available but due care should be subsequently exercised against allowing solutions to come in contact with the plugs since anticomplementary substances may be dissolved out.

3. The *pipettes* employed in complement-fixation tests should not be used for other purposes and it is advisable to have a 1 cc. pipette for each serum and spinal fluid, with enough 5 cc. pipettes for antigen, complement, corpuscles and hemolysin.

* Arthur H. Thomas Co., Catalogue No. 1724A.

All should be *physically and chemically clean* and perfectly dry; it is not necessary to sterilize them although this is advisable in a hot-air oven.

New pipettes should be carefully washed and dried before use to remove all traces of anticomplementary or hemolytic substances which may be present.

After use pipettes should be placed without delay in a cylinder or bucket of water in order to prevent serum or cells drying within the interiors. A wad of cotton in the bottom of the receptacle will prevent the chipping of the tips (which renders the last 0.1 cc. inaccurate), when pipettes are dropped in with more or less force.

They may be washed, placed in metal containers or wrapped in paper and dried in a hot-air oven in the same manner as described above for test-tubes.

It is ordinarily unnecessary to plug the mouth ends with cotton, although this is optional with the individual worker and advisable for the less experienced in the *art* of skilful pipetting. To the best of my knowledge there is no danger of infection from accidentally drawing syphilitic *serum* into the mouth. Fresh spinal fluids, however, may be infectious as shown by the frequency with which *Spirochæta pallida* has been found in rabbit testicle inoculation tests although I have never heard of the infection of laboratory workers in this manner. When the accident occurs it would appear to be sufficient to immediately and thoroughly wash out the mouth with diluted alcohol.

All pipettes should be clear, perfectly clean and free of all chipping. If they become discolored and especially by reason of sterilization without sufficient cleaning whereby serum or corpuscles become baked on the glass, they may be cleaned by complete immersion for one or two days in 2 per cent hydrochloric acid or bichromate cleaning fluid (2 parts potassium bichromate, 3 parts commercial sulphuric acid and 25 parts water), followed by thorough washing in running water to remove all traces of acid before sterilization.

PREPARATION OF SALINE SOLUTION.

The importance of saline solution in my tests has been emphasized in Chapter VIII because it is sometimes responsible for trouble with the hemolytic system. Whenever it is difficult or impossible to obtain a satisfactory unit of hemolysin or complement or both and one can be certain that the complement is sufficiently active and the corpuscles not unduly resistant, the saline solution should be suspected and especially if the hemolysin has been previously used with success. *Hemolysin rarely deteriorates* even when shipped over long distances although when difficulties develop with the

hemolytic system it is usually and erroneously first suspected as the cause of trouble.

In Philadelphia and most other localities perfectly satisfactory saline solution may be prepared with chemically pure sodium chloride and either distilled or tap water. In limestone districts distilled water is required. But with old distils in any locality heavily crusted with inorganic matter, the distilled water may be very unsatisfactory.

In the great majority of localities the following method is perfectly satisfactory:

(*a*) Weigh out 8.5 grams of dry chemically pure or a good grade of table sodium chloride kept in a tightly-stoppered bottle and dissolve in 1000 cc. of *freshly* distilled or freshly drawn tap water (I use tap water routinely). Do not use stored or stale distilled or spring water because of acidity and the presence of contaminating bacteria and molds.

(*b*) If not perfectly clear filter through paper.

(*c*) Plug the flasks with gauze covered cotton stoppers and sterilize by heating in an Arnold for one hour or autoclave for ten or fifteen minutes. The former is preferred in order to avoid excessive concentration of salt by loss of water in steam and excessive changes in hydrogen-ion concentration. Sterilization is not necessary if the solution is used at once.

Kilduffe has recommended preparing a 8.5 per cent solution and from this preparing the 0.85 per cent solution as needed by diluting with 9 volumes of distilled water.

A satisfactory saline solution should not be of itself hemolytic when 1 or 2 drops of washed corpuscles are added to 5 cc. of the solution in a test-tube followed by water-bath incubation for one hour. Neither should it be antihemolytic as determined by the hemolysin or complement titrations. The antisheep hemolysin for my test should give a unit of 0.5 cc. of a 1 to 4000 dilution at least when titrated with 0.3 cc. of 1 to 30 complement and 0.5 cc. of a 2 per cent suspension of washed sheep corpuscles with a water-bath incubation of one hour. It is possible that failure of hemolysis may be due to defective hemolysin or complement or to the use of corpuscles of increased resistance to serum hemolysis but whenever these factors may be excluded it is likely that the saline solution is defective.

If difficulties are experienced with saline prepared of distilled water, use ordinary tap water. If still unsatisfactory add 0.1 gm. of magnesium sulphate to each 1000 cc. as recommended by Kellogg (page 114) or add the buffers as recommended by Sanford (page 113).

PREPARATION OF ANTIGEN.

This is my new cholesterolized and lecithinized alcoholic extract of beef heart (designated as C. L.) described in Chapter XIV. The details of preparation follow, the original method of preparation

requiring about two weeks while the rapid method requires about two days, although occasional antigens may not be quite as high in antigenic activity as those prepared by the longer method; the original method therefore is to be preferred.

Original Method.—This is prepared of desiccated and finely powdered beef heart muscle. During the past three years I have used the Bacto Beef Heart prepared by the Digestive Ferments Company of Detroit with uniform success. The powder should be prepared of several hearts in order to be *polytropic* and the Bacto preparation is ordinarily the product of 100 or more organs.

1. *Fresh* beef hearts are washed free of blood, dissected free of fat and large bloodvessels and the muscle passed through a meat grinder three or four times. The minced tissue is then rapidly dried in a vacuum apparatus or equally well by spreading in *thin layers* on clean glass plates and drying by *rapid fanning*, preferably in a dust-proof box, for eighteen to twenty-four hours, turning the layers after the first ten to twelve hours. The cakes of dried material are now broken up, placed in an incubator over night and ground into a fine powder, which is kept in tightly-stoppered bottles of colored glass at room temperature. As previously stated *three or more hearts may be prepared at one time and a mixture of the powders used in preparing extracts. Rapid drying is essential to prevent decomposition* which results in greatly increasing the hemolytic activity of the extracts.

2. Twenty-five grams of powdered muscle are extracted with about 200 cc. of ether in a Sohxlet for eighteen hours; if this apparatus is not available, the powder is extracted with 200 cc. of ether in a tightly-stoppered bottle at room temperature for five days, being shaken occasionally each day. The ether is carefully removed and saved for the time being in a tightly-stoppered bottle.

3. The powder is now dried by fanning for a few minutes or by spreading on a glass plate for several hours, placed in a bottle and extracted with 200 cc. of 95 per cent alcohol in an incubator for four days.

4. The alcohol is carefully decanted, poured into a flat shallow dish and fanned dry. The residue is extracted with 30 to 50 cc. of ether for the ether-soluble portion; this ethereal extract is covered and allowed to stand for an hour or two for the heavy insoluble particles to settle out.

The ethereal solution is now mixed with the ether of primary extraction and the mixture concentrated by fanning until reduced to about one-quarter volume or about 25 to 30 cc. Six volumes or about 150 cc. of *pure* acetone are now added to the concentrated ether which throws down a whitish precipitate; the mixture is covered and placed aside for several hours or over night for the complete separation of the acetone-insoluble portions. On the following day the supernatant acetone is decanted and the sticky residue of acetone-insoluble lipoids removed and kept in acetone in a tightly-stoppered wide-mouthed bottle for future use.

5. The muscle powder is now extracted for a second time with 100 cc. of absolute acetone-free ethyl alcohol in an incubator for six days guarding against evaporation and shaking the mixture once or twice a day and if possible for at least one day in a mechanical shaker; the extract is now filtered through fat-free paper.

6. Two-tenths gram pure cholesterol and all of the acetone-insoluble lipoids previously prepared are dissolved in 10 to 20 cc. of pure ether and the cloudy brownish mixture slowly added to the filtered alcoholic extract and well shaken. The extract is now placed in the incubator for a few hours

or over night, being shaken occasionally, and then at room temperature for a day or two; the light brownish precipitate is now removed by filtration through fat-free paper or by decanting the extract. The finished antigen is kept in a tightly-stoppered brown glass bottle in the laboratory. Any precipitate forming after this time is left undisturbed.

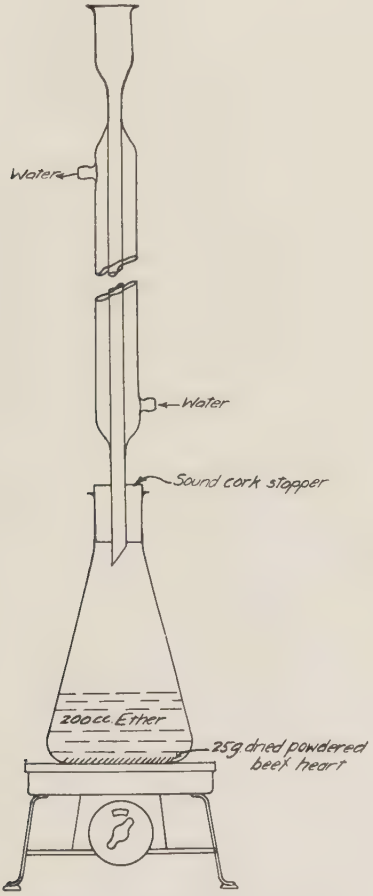

FIG. 44.—An electrically heated and condenser arrangement for the rapid preparation of antigen.

Rapid Method.—1. Place 25 grams of the muscle powder along with 200 cc. of ether in an Erlenmeyer flask fitted with a Liebig's condenser as shown in Fig. 44. With an electric heater* boil for two hours.

2. Decant off the ether and save. Place the powder in an evaporating dish and dry in an incubator (ordinarily a few minutes). Return the powder to the same flask, add 200 cc. of ethyl alcohol (95 to 99 per cent) and boil for two hours.

* Arthur H. Thomas Catalogue No. 6151 is satisfactory.

3. Decant off the alcohol and evaporate to dryness in an evaporating dish. Extract the residue with 50 cc. of ether and add to the ether of first extraction. Evaporate the mixture to about 25 to 30 cc. and add 6 volumes of chemically pure acetone (150 cc.). Stand aside for the precipitate to settle out. Decant and discard the acetone but save the residue of acetone-insoluble lipoids.

4. To the powder in the flask add 100 cc. of absolute acetone-free ethyl alcohol and boil for two hours. Filter off this secondary alcoholic extract and discard the powder.

5. Dissolve the residue of acetone-insoluble lipoids and 0.2 grams of pure cholesterol in 10 cc. of ether. Add to the secondary alcoholic extract. Shake well and place in an incubator for a few hours and then *keep at room temperature.* Filter after a day or two and titrate.

PREPARATION OF CORPUSCLE SUSPENSION.

For many years I have used sheep blood obtained from abattoirs with uniform success and during the past several years have collected and preserved it with formalin by the following method:

In a clean (but not necessarily sterile) quart-sized mason jar place 30 cc. of a 10 per cent solution of sodium citrate in saline solution and 2 cc. of formalin. At the abattoir have the jar almost filled with *fresh* blood (blood kept over in buckets is unsatisfactory), screw on the top, mix well with the citrate-formalin solution and keep in a refrigerator. Ordinarily it is fit for use over a period of two to three weeks at least but as soon as the corpuscles become too fragile a fresh supply should be secured.

If preservation is not desired glass beads alone may be placed in the jar and after filling with blood thoroughly shaken for defibrination. Blood collected in this manner will keep at a low temperature (jar placed on a block of ice) for about a week. If beef blood is used it may be collected by either method.

Otherwise one or more sheep may be kept and bled as required. But in my opinion this is a useless expense if abattoir blood can be secured at least once every week or two. Small amounts of blood may be obtained from an ear vein and larger amounts from an external jugular by the following method:

1. One may do the bleeding alone, although the aid of an assistant is usually necessary, especially if the animal is large and vicious.

2. The sheep is thrown on its back, and the head is held on the knees of an assistant seated on a low box or stool.

3. The operator may straddle the animal to hold down the fore feet, although this is not necessary unless the animal is vicious.

4. The wool on the left side of the neck is clipped closely with scissors and alcohol applied.

5. The operator then grasps the neck low down with the left hand, and by means of the thumb exerts pressure over the base of the neck. The external jugular vein will be found in a groove between the omohyoid and sterno-cleido-mastoid muscles (Fig. 45). Firm pressure over the base of the neck usually distends the vein, which may be seen or easily felt. After

locating the vein the pressure should be released for an instant, when the distention will disappear. In this way the operator may be more certain that he has located the vein.

6. A sterile stout needle, at least 2 inches in length and provided with a trocar and special shank for firm grasping, is passed quickly into the distended vein in an upward and inward direction. It is essential that the needle be sharp, otherwise it will be turned aside by the wall of the vein.

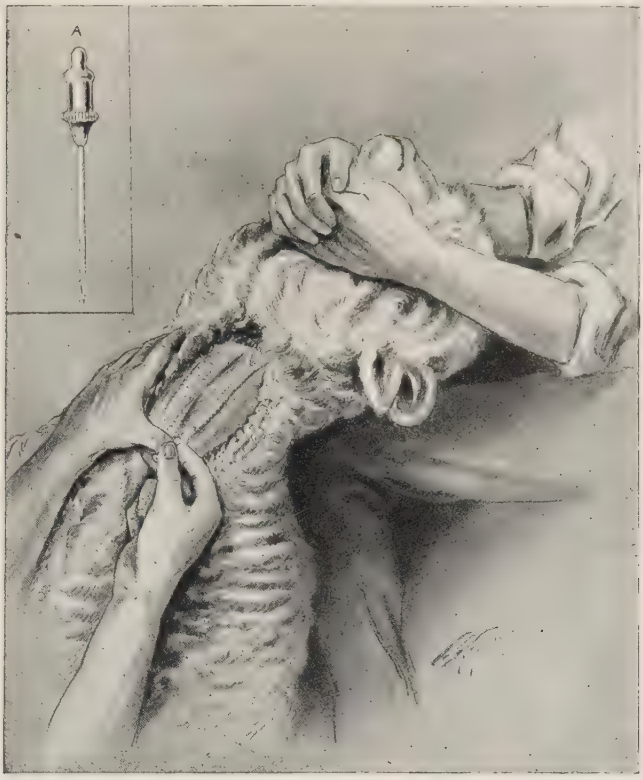

FIG. 45.—Method of bleeding a sheep from the external jugular vein. The operator is distending the vein by pressure over the base of the neck with the left hand. When distended, the vein can usually be felt beneath the skin. The needle here shown is reduced to a little more than one-half the actual size. (From Kolmer: Infection, Immunity and Biologic Therapy, W. B. Saunders Co., Publishers.)

The end of the needle must not have too long a bevel, or the point will pierce the opposite wall before the body of the needle is well within the vein. The trocar is now removed, and blood collected in a flask or bottle and defibrinated with glass beads and rods. A short piece of rubber tubing may be attached to the needle. A suction apparatus is not needed because the flow of blood is good so long as pressure is preserved over the vein at the base of the neck.

7. When the required amount of blood has been secured, pressure is released and the needle quickly withdrawn. Bleeding ceases at once, and the neck is then washed with alcohol.

8. By this method the same vein may be used over and over again for several years. I have never known infection to occur, although the gradual formation of scar tissue about the site of puncture may interfere with the operation.

The method of washing the corpuscles and preparing the suspension is as follows:

1. A satisfactory electric centrifuge should be available. The instrument should be started and stopped slowly.

2. Washing the blood is best accomplished in accurately graduated centrifuge tubes. Two sizes of centrifuge tubes, especially recommended for this work, are shown in Fig. 38. The smaller tube fits the ordinary 15 cc. shield and is used for washing small amounts of blood when 2 cc. or less of massed corpuscles are required; the larger tube is made to fit the 50 cc. shield of the International centrifuge and is used when larger amounts of blood are required. Both are graduated in 0.1 cc. and permit of an accurate measure of the corpuscle mass after the final washing.

The centrifuge tubes may be sterilized prior to use, but this is not necessary; they should be chemically clean and rinsed with saline solution before use.

3. In washing defibrinated or citrated-formalized blood, 1 volume of blood is placed in a centrifuge tube, 2 volumes of saline solution added and gently mixed. Each tube must be accurately counterbalanced in the centrifuge and whirled until all corpuscles have been thrown down, the time required depending upon the speed of the centrifuge (first washing).

4. The supernatant fluid is carefully removed down to the corpuscle mass with a capillary pipette (attached to a suction pump as shown in Fig. 46 or by means of a rubber teat) and at least 3 to 5 volumes of saline solution added; by capping the tube and inverting, all the corpuscles are thoroughly but gently stirred and mixed in the saline solution and the tubes again centrifuged (second washing).

5. The supernatant fluid is again removed, replaced with saline solution, the corpuscles thoroughly mixed and again centrifuged for *twice as long* as found necessary to throw down the corpuscles in the second washing. This longer period of centrifuging is advisable to firmly and evenly pack the washed cells and the *speed and duration of centrifuging should be uniform in each laboratory as based upon experience with the particular centrifuge in use.*

6. If the supernatant fluid is discolored with hemoglobin the cells should be washed again until the supernatant fluid is practically colorless.

7. With the last washing the centrifuge is stopped slowly so as not to disturb the corpuscle mass, and *the volume of corpuscles read in the centrifuge tube before removing the supernatant fluid.* The

latter is now removed and a suspension of proper strength prepared. A 2 per cent suspension for example, is prepared by washing the cells from the centrifuge tube into a proper flask with forty-nine times as much saline solution as corpuscle mass.

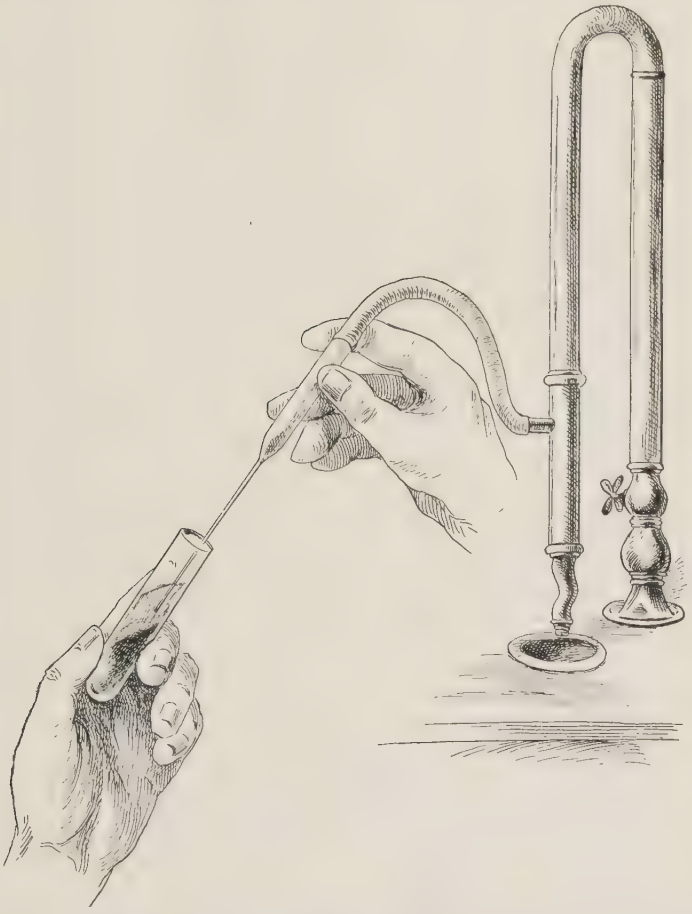

FIG. 46.—A suction pump. By attaching a capillary pipette to the rubber tubing fluid may be removed without disturbing the sediment. (From Kolmer: Infection, Immunity and Biologic Therapy, W. B. Saunders Co., Publishers.)

8. As the corpuscles in suspension tend to settle rapidly, the suspension should be *gently and thoroughly shaken* by hand before being used and *during the time of use*, if several minutes are required for pipetting the suspension; otherwise the doses will be uneven and inaccurate.

9. So far as practicable the suspension should be kept in a refrigerator when not being used.

I have found it advisable to prepare at once a sufficient amount of suspension for the titrations and complement-fixation tests since in my experience the suspension can be readily kept over night in a refrigerator without hemolysis. Reyner[5] however, advises making up only sufficient of a 2 per cent suspension for the hemolysin and complement titrations and to put away in a refrigerator the washed corpuscles, making up next day a fresh 2 per cent suspension for the complement-fixation tests, since in his experience a suspenison of corpuscles carried over to next day may show slight hemolysis. Either method may be employed.

PREPARATION OF HEMOLYSIN.

As stated in Chapter XII antisheep hemolysin is preferred and it is readily and easily prepared by the immunization of large, healthy and previously unused rabbits; *white rabbits are preferred.* My method is to give an intravenous injection of 5 cc. of a 10 per cent suspension of washed corpuscles every five days for five or six injections. Antiox hemolysin is second choice but is somewhat more difficult to prepare and eight to ten injections may be required. Another good method is to give 1 cc. of a 10 per cent suspension daily for three weeks or 10 cc. of a 1 per cent twice a week for eight to ten injections. It is well to immunize 3 or more rabbits at a time in case 1 or 2 succumb before immunization is completed. The technique of intravenous injection is as follows:

1. The posterior auricular vein along the outer margin of the ear is the one of choice.
2. Commence as near the tip of the ear as possible and subsequent injections may be given nearer and nearer the root.
3. The animal should be held firmly, as the slightest movement may result in piercing the vein through and through and require reinsertion of the needle. This is accomplished satisfactorily by placing the rabbit upon the edge of the table and holding it firmly there by grasping the neck and front quarters, the assistant at the same time compressing the root of the ear with the thumb and forefinger.
4. If the hair is long, clip it.
5. The ear is struck gently with the fingers and washed with alcohol and xylol; the friction will render the vein more conspicuous.
6. The ear is grasped at its tip and stretched toward the operator, or the vein may be steadied by rolling the ear gently over the left index finger and holding it between the finger and thumb.
7. The inoculum should be free from solid particles and all the air excluded from the syringe.
8. Hold the syringe as one would hold a pen, and thrust the point of the needle through the skin and the wall of the vein until it enters the lumen of the vein (Fig. 47).

The wooden box shown in Fig. 48 is very convenient for holding rabbits for intravenous injection or for bleeding from the ears.

9. Direct the assistant to release the pressure at the root of the ear, and *slowly* inject the inoculum. If the fluid is being forced into the subcutaneous tissue, which will be evident at once by the swelling which occurs, the injection must cease and another attempt be made.

FIG. 47.—Intravenous inoculation of a rabbit. (From Kolmer: Infection, Immunity and Biologic Therapy, W. B. Saunders Co., Publishers.)

10. The needle is quickly withdrawn, a small piece of cotton moistened with alcohol placed upon the puncture wound, and firm compression applied. *Wash the ear thoroughly with alcohol and water to remove xylol, otherwise a low-grade inflammation will follow, which will render subsequent injections more difficult.*

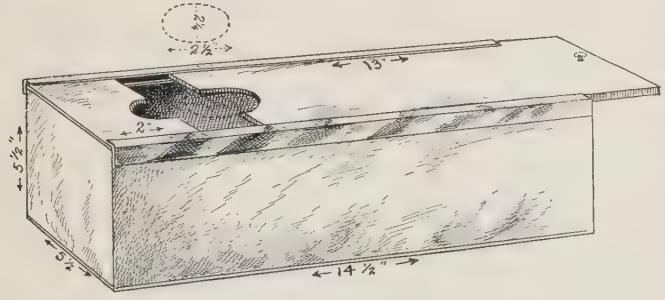

FIG. 48.—Wooden box for holding rabbits for intravenous injection. (From Kolmer: Infection, Immunity and Biologic Therapy, W. B. Saunders Co., Publishers.)

One week after the last injection a small amount of blood may be obtained from an ear vein as follows:

1. Flip an ear vigorously with the hand, and rub with xylol and alcohol. the xylol produces marked congestion and afterward should be carefully

removed with alcohol and water, as it produces a low-grade inflammatory reaction.

2. Puncture a marginal vein with a large needle. The blood will flow quickly in drops and practically any amount up to 10 cc. or even more may be collected in a centrifuge or test tube (Fig. 49). For making preliminary tests of serum during immunization 2 cc. of blood is usually sufficient. Bleeding may be checked by making firm pressure over the puncture.

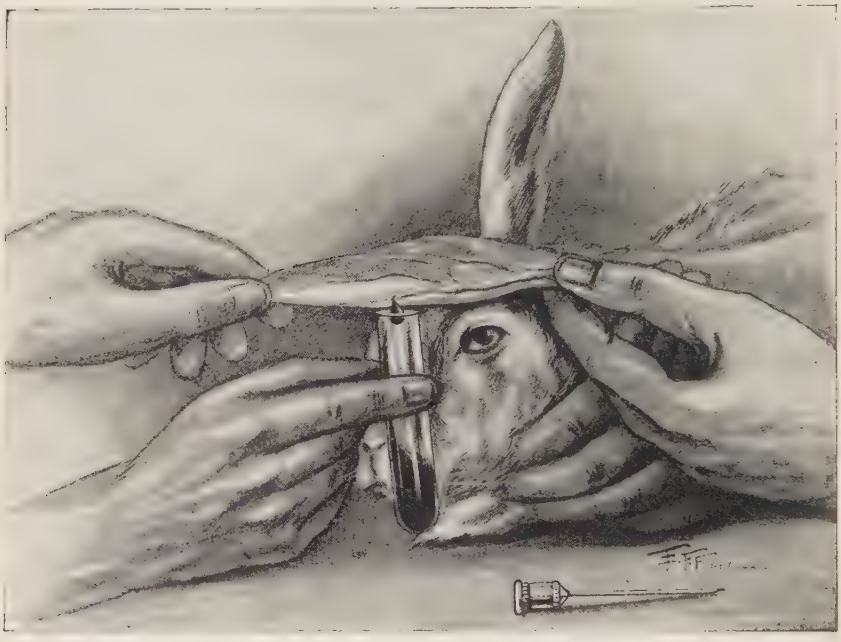

FIG. 49.—Method of bleeding a rabbit from the ear. A slit is made in the marginal vein. (From Kolmer: Infection, Immunity and Biologic Therapy, W. B. Saunders Co., Publishers.)

The serum is separated, heated at 55° C. for thirty minutes and titrated as described on page 391. The unit of antisheep hemolysin should be at least 0.5 cc. of 1 to 4000; as a general rule it is much higher. If an antiox hemolysin is being prepared the unit should be at least 0.5 cc. of 1 to 2000. In either case if the titers are less than these amounts the rabbits should receive two or three additional injections and be retitrated.

If satisfactory the animal should be bled to death in such manner as to secure the maximum amount of blood. My method is to moisten the fur of the neck with alcohol, stun the animal with a sharp blow on the forehead or to anesthetize with ether and to sever the great vessels with a pair of sharp scissors or a razor, the blood being collected in centrifuge tubes by means of a funnel as

shown in Fig. 50 for the bleeding of guinea-pigs. It is well to have an assistant hold and elevate the hind legs. Even though hairs and bacteria gain access to the blood no harm is done since the serum is preserved with an equal amount of glycerin which is immediately antiseptic and slowly bactericidal. Attempts to secure the blood aseptically from a carotid artery frequently fail and are unnecessary.

The blood is now allowed to coagulate and the serum to separate in a refrigerator when the clear serum is pipetted off. My method, however, is to break up each clot after coagulation with a glass rod and centrifuge thoroughly. This yields more serum although it is usually deeply tinged with hemoglobin from broken up corpuscles, which, however, does no harm.

To the mixed and *unheated* sera is now added an equal amount of chemically pure glycerin. This gives a 50 per cent concentration which preserves the serum against bacterial growths and keeps it almost indefinitely at ordinary refrigerator temperatures. The titer generally drops slightly at once and then holds remarkably constant.

PREPARATION OF COMPLEMENT.

As stated in Chapter XI a mixture of freshly prepared guinea-pig sera is employed for complement. The serum may be preserved for about three weeks as described below and gives satisfactory results but fresh serum is to be preferred. The mixed sera of at least 3 or more guinea-pigs should be used (the more the better) and in small laboratories the waste of serum may be avoided by bleeding 3 or more animals from the heart or by preserving the excess. The methods which I employ are as follows:

1. Select large well-nourished animals *which have not been fed within twelve to twenty-four hours of the time for bleeding; avoid pregnant animals.*

2. Collect blood in a *chemically clean* and preferably sterile centrifuge tube by means of a clean funnel about 4 inches in diameter as shown in Fig. 50 or in a Petri dish. I prefer the former because if separation of serum is unsatisfactory the clot may be gently broken up with a glass rod and the serum secured by centrifuging the material. Chemical cleanliness is essential because traces of acids or alkalies are destructive for complement.

3. If guinea-pigs are to be bled to death, stunning the animals with a blow at the base of the skull is preferable to ether and chloroform anesthesia because cardiac activity is not interfered with and the maximum amount of blood is obtained. Guinea-pigs may also be bled from the ears without anesthesia; or from the heart under light ether anesthesia as follows: A 5 cc. syringe fitted with a No. 20 or 22 needle is employed. The animal is fastened to a board or held by an assistant and lightly anesthetized. The point of maximum pulsation is determined and the needle slowly entered into the right chambers of the heart. As a general rule, 2 to 5 cc. of blood may be obtained by gentle suction, the amount depending upon the size of the animal. Large male animals are recommended and may be used repeatedly. After withdrawal of the needle the animal rapidly recovers, although occasionally bleeding may follow into the pericardial sac (Fig. 51).

4. In bleeding a guinea-pig to death it is not necessary to shave the neck; a few hairs in the blood do not alter the properties of the serum. The great vessels on one or both sides should be *quickly* severed and preferably with a

FIG. 50.—Method of bleeding a guinea-pig. (From Kolmer: Infection, Immunity and Biologic Therapy, W. B. Saunders Co., Publishers.)

pair of stout sharp scissors with one pointed blade to facilitate piercing of the skin and underlying tissues. It is easy to avoid cutting the trachea and esophagus, but it does not appear to make any difference whether the trachea is cut; the esophagus should not be cut because in squeezing the abdomen to pump out blood in the great vessels stomach contents may escape, although even this accident is rare in our experience.

5. *Do no use the blood immediately after bleeding.* If the animals are bled on the day the serum is to be used, place the clots in the incubator at 37° C. for an hour followed by breaking up each with a glass rod and centrifuging, or let the clots stand at room temperature for two or three hours for separation of serum which if not

FIG. 51.—Method of securing blood from heart of a guinea-pig. (From Kolmer: Infection, Immunity and Biologic Therapy, W. B. Saunders Co., Publishers.)

complete may be finished by centrifuging. Animals may be bled in the afternoon of the day before and the clots placed in the incubator for one hour or left at room temperature for two hours and then placed in a refrigerator until the following day. If the sera have separated but poorly, gently break up the clots and centrifuge. *It is preferable to leave the serum on the clot in the refrigerator until ready for use; unused serum should be returned to the refrigerator until required.* Traces of hemoglobin in the serum do not interfere, but the serum should be free of corpuscles.

6. If the work at hand on any day does not require all the serum of three or more pigs it is better to bleed small amounts of blood

from the hearts of several large pigs than to use the serum of one animal. If this is not possible, I am convinced that the use of the preserved sera of three or more pigs is better under certain conditions than the use of the fresh serum of a single animal. The following *method for preservation* can be recommended:

Add 0.3 gm. of chemically pure sodium chloride to each cubic centimeter of serum and store in a tightly-stoppered dark glass bottle or 1 cc. amber-colored ampoules in a refrigerator. If an ice refrigerator is used the bottle or ampoules should be wrapped in a towel and kept on a block of ice. In an electric refrigerator they may be kept in the compartments used for preparing cubes of ice. For use 1 cc. of the serum is diluted with 29 cc. of water which gives a 1 to 30 isotonic solution as used in my test. Growths of bacteria in the serum hastens deterioration. This may be prevented by adding to each cubic centimeter 0.05 cc. (approximately 1 drop) of a 5 per cent solution of tricresol or phenol, but is not required if the serum-salt mixture is kept at or near 0° to 2° C. As stated in Chapter XI preserved complement loses first in fixability and even if hemolytic activity is good (unit 0.3 to 0.4 cc. of 1 to 30) at the end of three weeks I believe it inadvisable to use it beyond this period.

PREPARATION OF SERA.

The Drummond mailing case opener* shown in Fig. 52 is very useful. All specimens should be kept in a refrigerator until ready for testing as a safe-guard against the acquisition of anticomplementary properties and all should be carefully labelled and numbered to prevent possible confusion and mistakes.

It is now my custom to *routinely* remove the natural antisheep hemolysins from *all sera*. This makes it necessary to centrifuge each but by using a multiple centrifuge holder as shown in Fig. 53† 24 specimens may be centrifuged at one time since each of the 8 holders will carry 3 tubes with outside diameters up to 16 mm. If smaller tubes are employed the Laird multiple holder shown in Fig. 54‡ may be employed since it carries 12 tubes and enables one to centrifuge 96 specimens at a time. The speed is not lessened or the work of the motor materially increased with a shielded holder of this kind. The technique is as follows:

1. All specimens are lined up and properly labelled.

2. To each is added *a drop of washed corpuscle sediment for approximately each 2 cc. of blood and serum* as gauged by inspection.

* Marketed by Edward P. Dolbey & Co., Philadelphia Pa.

† Arthur H. Thomas Co. Catalogue No. 3053 and 3054 accommodates tubes with diameters up to 16 mm.

‡ Marketed by George H. Wahmann, Baltimore, Md. The middle eight squares carries tubes with outside diameters of 14 mm.; the four corner squares, tubes of 10 mm.

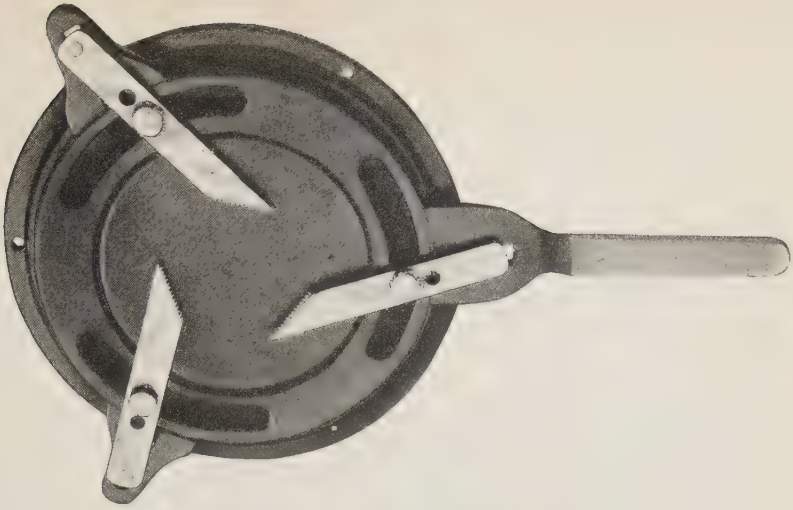

FIG. 52. -The Drummond mailing case opener.

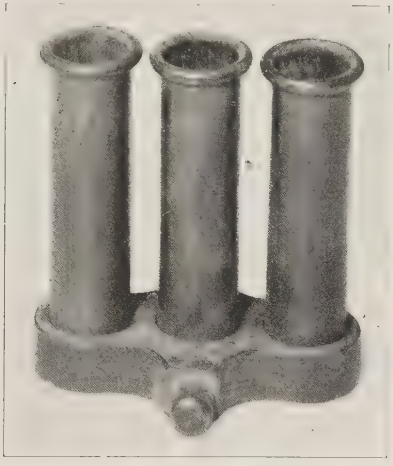

FIG. 53.—Multiple centrifuge tube holder.

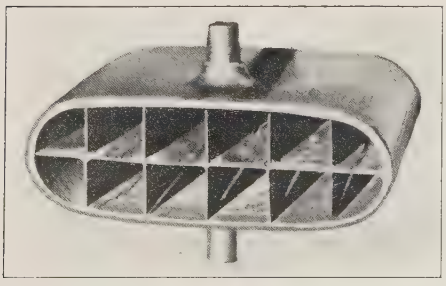

FIG. 54.—The Laird multiple centrifuge tube holder.

3. Each specimen is then thoroughly mixed with a *perfectly clean* glass rod (need not be necessarily sterile).

4. All are then placed in a *refrigerator for five minutes* to enable the sheep corpuscles to absorb the hemolysins with none or but a minimum and harmless amount of hemolysis.

5. All specimens are now centrifuged with due care to have the centrifuge properly balanced by carefully counterbalancing each pair of centrifuge tube holders with scales as shown in Fig. 55 and placing the holders opposite to each other in the centrifuge. For nice adjustment of balance small glass beads are convenient.

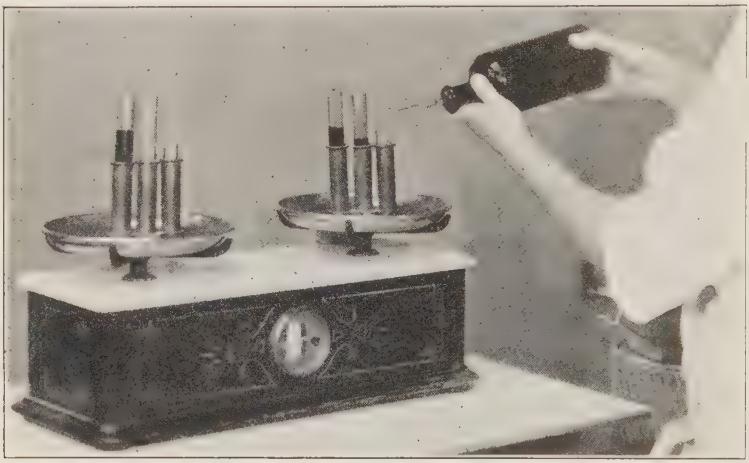

FIG. 55.—Method of counterbalancing centrifuge tubes.

6. The sera are now separated into test-tubes properly labelled. Some of the sera may be tinged with hemoglobin but this does no harm.

7. The tubes are now placed in a water-bath at 55° C. for *fifteen minutes* when they are ready for testing.

PREPARATION OF SPINAL FLUIDS.

These are usually tested without any preliminary preparation as they do not contain enough natural antisheep hemolysin to require removal or enough complement to require inactivation by heating at 55° C. If a specimen contains considerable blood which has not had time to settle out it should be centrifuged but otherwise no preparation is required as spinal fluids are tested as delivered without preliminary heating unless they are more than a week old when they may be heated at 55° C. for fifteen minutes to remove thermolabile anticomplementary substances.

DAILY TITRATION OF HEMOLYSIN AND COMPLEMENT.

As stated in Chapter XIII it is advisable to titrate both the hemolysin and complement before each days' work. The hemolysin could be used in a dose of 2 units as determined by previous titrations since it keeps so well in a refrigerator over long period of time but I recommend and practice its daily titration *in order to adjust for any natural antisheep hemolysin apt to be present in the complement serum;* under these conditions it is entirely unnecessary to exclude complement serum containing natural hemolysin or to remove the hemolysin beforehand.

Dilute 0.2 cc. of complement with 5.8 cc. of saline solution which gives sufficient of 1 to 30 dilution for both titrations. If preserved complement is being employed dilute 0.2 cc. with 5.8 cc. of distilled water. Keep the balance of undiluted serum in the refrigerator.

Titration of Hemolysin.—1. Arrange a series of 10 test-tubes and place 0.5 cc. of varying dilutions of hemolysin in each tube, respectively.

2. Ordinarily a range of dilutions from 1 to 1000 to 1 to 16000 is sufficient but depending upon the hemolytic activity of the complement and resistance of the corpuscles higher or lower dilutions may be required. A 1 to 100 dilution preserved with 0.2 per cent phenol against bacterial contamination may be prepared as follows and kept in a refrigerator for several weeks, from which the higher dilutions are prepared as needed:

Equal parts hemolytic serum and glycerin	2 cc.
Physiological saline	94 cc.
5 per cent phenol in saline or water	4 cc.

3. For titration dilute to 1 to 1000 (0.5 cc. of 1 to 100 + 4.5 cc. saline solution) and in a series of 10 test tubes prepare dilutions as follows:

1. 0.5 cc. of 1 to 1000 = 1 to 1000
2. 0.5 cc. of 1 to 1000 + 0.5 cc. saline = 1 to 2000
3. 0.5 cc. of 1 to 1000 + 1.0 cc. saline = 1 to 3000
4. 0.5 cc. of 1 to 1000 + 1.5 cc. saline = 1 to 4000
5. 0.5 cc. of 1 to 1000 + 2.0 cc. saline = 1 to 5000
6. 0.5 cc. of 1 to 3000 + 0.5 cc. saline = 1 to 6000
7. 0.5 cc. of 1 to 4000 + 0.5 cc. saline = 1 to 8000
8. 0.5 cc. of 1 to 5000 + 0.5 cc. saline = 1 to 10000
9. 0.5 cc. of 1 to 6000 + 0.5 cc. saline = 1 to 12000
10. 0.5 cc. of 1 to 8000 + 0.5 cc. saline = 1 to 16000

Mix contents of each tube very thoroughly.

4. Use 0.5 cc. of each dilution in test-tubes (85 x 15 mm.) and to each add 0.3 cc. of 1 to 30 dilution of the same complement and 0.5 cc. of a 2 per cent suspension of the same corpuscles as used in the complement-fixation tests; add 1.7 cc. saline to each tube to make the total volume in each 3 cc.

5. Mix the contents of each tube and place in the water-bath at 38° C. for one hour; *the unit is the highest dilution of hemolysin showing just complete and sparkling hemolysis free of opacity. Two units are employed in the titration of complement and antigen and in the complement-fixation tests.*

Table 55 shows the ensemble and results of a titration.

TABLE 55.—TITRATION OF HEMOLYSIN.

Tube.	Hemolysin.	Complement, 1:30.	Corpuscles (2 per cent).	Saline.	After water-bath incubation for one hour.
1	0.5 cc. 1:1000	0.3 cc.	0.5 cc.	1.7 cc.	Complete hemolysis.
2	0.5 cc. 1:2000	0.3 cc.	0.5 cc.	1.7 cc.	Complete hemolysis.
3	0.5 cc. 1:3000	0.3 cc.	0.5 cc.	1.7 cc.	Complete hemolysis.
4	0.5 cc. 1:4000	0.3 cc.	0.5 cc.	1.7 cc.	Complete hemolysis.
5	0.5 cc. 1:5000	0.3 cc.	0.5 cc.	1.7 cc.	Complete hemolysis.
6	0.5 cc. 1:6000	0.3 cc.	0.5 cc.	1.7 cc.	*Complete hemolysis; unit.*
7	0.5 cc. 1:8000	0.3 cc.	0.5 cc.	1.7 cc.	Marked hemolysis.
8	0.5 cc. 1:10000	0.3 cc.	0.5 cc.	1.7 cc.	Marked hemolysis.
9	0.5 cc. 1:12000	0.3 cc.	0.5 cc.	1.7 cc.	Slight hemolysis.
10	0.5 cc. 1:16000	0.3 cc.	0.5 cc.	1.7 cc.	No hemolysis.

In the above titration the unit was 0.5 cc. of 1 to 6000; 2 units were contained in 0.5 cc. of 1 to 3000.

6. Sufficient hemolysin is now prepared for the complement titration and complement-fixation tests so that 2 units are contained in 0.5 cc. For example, in the above titration 5 cc. of 1 to 100 hemolysin diluted with 145 cc. of saline solution would give 150 cc. of 1 to 3000 or sufficient for 300 tubes each carrying 2 units in a dose of 0.5 cc. If any is left over it will keep in the refrigerator.

Titration of Complement.—1. Arrange 10 test tubes and place 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 cc. of 1 to 30 complement in each respectively; the tenth tube serves as a corpuscle control for this and the hemolysin titration.

2. Into each of the first 9 tubes place 10 units of antigen so diluted that this amount is contained in 0.5 cc. This dilution is prepared by placing the required amount of saline solution in a flask and adding antigen drop by drop. Shake the flask after each addition of antigen. Prepare enough for the complement titration and the main tests. For example, if the dose of 10 units of antigen is 0.5 cc. of 1 to 300 dilution, place 150 cc. of saline in a flask and add 0.5 cc. of antigen drop by drop to give approximately 150 cc. or sufficient for 300 tubes.

3. Place sufficient saline solution in each tube to make the total volume about 2 cc.

4. Mix contents of each tube and place in a water-bath at 38° C. for one hour.

5. Add 0.5 cc. hemolysin (2 units) and 0.5 cc. corpuscle suspension (2 per cent) to each tube; mix and reincubate one hour.

6. Ordinarily the smallest amount of 1 to 30 complement giving *complete and sparkling hemolysis* is taken as the unit but *experience has shown that with the method of primary incubation employed in the complement-fixation tests namely, fifteen to eighteen hours at 6° to 10° C. followed by ten to fifteen minutes in a water-bath at 38° C. this is insufficient; in this test the unit is taken as the amount of complement in the next higher tube.* For example, if hemolysis is just complete with 0.25 cc. the unit is taken as 0.3 cc. and double this amount used for the antigen titrations and complement-fixation tests. For convenience I have designated this amount as *2 full units of complement.* Experience has shown that the reactions are apt to be unsatisfactory if less than 0.4 cc. of 1 to 30 complement is employed; occasionally hyperactive sera yield a unit with 0.1 cc. of 1 to 30 but when this occurs it is necessary to use 0.4 cc. for the dose of complement.

Table 56 shows the ensemble, the results of a titration and the method of reading:

TABLE 56.—TITRATION OF COMPLEMENT.

Tube.	Complement, 1:30.	Antigen (10 units).	Saline.		Hemolysin (2 units).	Corpuscles (2 per cent).	After water-bath incubation for one hour.
1	0.1 cc.	0.5 cc.	1.4 cc.	Water-bath 38° C. for one hour.	0.5 cc.	0.5 cc.	No hemolysis.
2	0.15 cc.	0.5 cc.	1.4 cc.		0.5 cc.	0.5 cc.	Slight hemolysis.
3	0.2 cc.	0.5 cc.	1.3 cc.		0.5 cc.	0.5 cc.	Marked hemolysis.
4	0.25 cc.	0.5 cc.	1.3 cc.		0.5 cc.	0.5 cc.	Complete hemolysis; the exact unit.
5	0.3 cc.	0.5 cc.	1.2 cc.		0.5 cc.	0.5 cc.	*Complete hemolysis; the full unit.*
6	0.35 cc.	0.5 cc.	1.2 cc.		0.5 cc.	0.5 cc.	Complete hemolysis.
7	0.4 cc.	0.5 cc.	1.1 cc.		0.5 cc.	0.5 cc.	Complete hemolysis.
8	0.45 cc.	0.5 cc.	1.1 cc.		0.5 cc.	0.5 cc.	Complete hemolysis.
9	0.5 cc.	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete hemolysis.
10	..	..	2.5 cc.		..	0.5 cc.	No hemolysis (control).

In practice the hemolysin titration may be placed in the water-bath at the same time as the complement titration; at the end of the first incubation of the complement titration the unit of hemolysin is available and 2 units added to all tubes of the complement titration, etc.

7. *Each 2 full units of complement are diluted with sufficient saline solution to make 1 cc. and called the dose of complement;* for example, if 0.3 cc. of 1 to 30 complement is the full unit the dose is 0.6 cc.

A convenient scheme for diluting the dose of complement to 1 cc. is as follows: divide 30 by the dose = the dilution to employ in dose of 1 cc.

For example:

Exact unit = 0.25 cc. of 1 to 30 dilution.
Full unit = 0.30 cc. of 10 to 30 dilution.
Two full units = 0.60 cc. of 10 to 30 dilution.

$$\frac{30}{0.6} = 50$$ or dilution of 1 to 50 in dose of 1 cc.

If 300 doses of complement were to be provided this would require 300 cc. of 1 to 50, prepared by diluting 6 cc. guinea-pig serum with 294 cc. of saline solution. If preserved complement is employed a 1 to 30 dilution is first prepared by diluting 1 part of serum with 29 parts of distilled water. From this the final dilution is prepared with saline. For example, if the dose were 0.5 cc. of 1 to 50 diluting 180 cc. of 1 to 30 with 120 cc. of saline would give 300 cc. of 1 to 50 or sufficient for 300 tubes. These examples illustrate the method.

TITRATION OF ANTIGEN.

The new antigen should be kept at room temperature or in a refrigerator and titrated after preparation; repeat titrations every four to six months are advisable and more frequently if it shows evidence of losing in antigenic activity or acquiring increased anti-complementary activity. *The antigen should be diluted by placing the required amount of saline solution in a test-tube or Erlenmeyer flask and adding the required amount of extract drop by drop or in amounts of 0.1 cc. and shaking by rotating after each addition.*

In a series of 8 test-tubes prepare the following dilutions of antigen:

0.5 cc. antigen to 1.5 cc. saline = 1 to 4
0.5 cc. antigen to 2.0 cc. saline = 1 to 5
0.5 cc. antigen to 2.5 cc. saline = 1 to 6
1.0 cc. antigen 1 to 4 to 1.0 cc. saline = 1 to 8
1.5 cc. antigen 1 to 5 to 1.5 cc. saline = 1 to 10
1.0 cc. antigen 1 to 6 to 1.0 cc. saline = 1 to 12
1.0 cc. antigen 1 to 8 to 1.0 cc. saline = 1 to 16
1.0 cc. antigen 1 to 10 to 1.0 cc. saline = 1 to 20

Hemolytic Titration.—1. In a series of 8 regulation test-tubes place 0.5 cc. of the above dilutions of antigen, respectively.

2. To each tube add 0.5 cc. of a 1 to 10 dilution of normal human serum previously heated for fifteen minutes at 55° C. and 1.5 cc. of saline solution.

3. Mix the contents of each tube and place in a refrigerator at 6° to 10° C. for fifteen to eighteen hours followed by ten minutes in a water-bath at 38° C.

4. Add 0.5 cc. of 2 per cent corpuscles suspension to each tube; mix and place in a water-bath at 38° C. for one hour.

5. Allow tubes to stand several hours in a refrigerator and read the results; *the smallest amount of antigen just beginning to produce hemolysis is the hemolytic unit.* As a general rule there is no hemolysis at all even in the 1 to 4 dilution.

Table 57 shows the ensemble, the results of a titration and the method of reading:

TABLE 57.—HEMOLYTIC TITRATION OF ANTIGEN.

Tube.	Antigen 0.5 cc.	Heated human serum 1:10.	Saline.		Corpuscles (2 per cent).	Water-bath one hour.
1	1:4	0.5 cc.	1:5 cc.	Refrigerator 6° to 10° C. for fifteen to eighteen hours followed by water-bath for ten minutes.	0.5 cc.	No hemolysis.
2	1:5	0.5 cc.	1:5 cc.		0.5 cc.	No hemolysis.
3	1:6	0.5 cc.	1:5 cc.		0.5 cc.	No hemolysis.
4	1:8	0.5 cc.	1:5 cc.		0.5 cc.	No hemolysis.
5	1:10	0.5 cc.	1:5 cc.		0.5 cc.	No hemolysis.
6	1:12	0.5 cc.	1:5 cc.		0.5 cc.	No hemolysis.
7	1:16	0.5 cc.	1:5 cc.		0.5 cc.	No hemolysis.
8	1:20	0.5 cc.	1:5 cc.		0.5 cc.	No hemolysis.

Anticomplementary Titration.—1. In the first 8 tubes of a second series of 10 tubes place 0.5 cc. of the above dilutions of antigen, respectively.

TABLE 58.—ANTICOMPLEMENTARY TITRATION OF ANTIGEN.

Tube.	Antigen. 0.5 cc.	Heated normal serum* 1:10	Complement (2 full units).		Hemolysin (2 units).	Corpuscles (2 per cent).	Water-bath one hour.
1	1:4	0.5 cc.	1.0 cc.	Refrigerator 6° to 10°C. for fifteen to eighteen hours followed by water-bath for ten minutes.	0.5 cc.	0.5 cc.	Slight inhibition of hemolysis.
2	1:5	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	*Slight inhibition of hemolysis (unit).*
3	1:6	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete hemolysis.
4	1:8	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete hemolysis.
5	1:10	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete hemolysis.
6	1:12	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete hemolysis.
7	1:16	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete hemolysis.
8	1:20	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete hemolysis.
9	0.5 saline	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete hemolysis.
10	1.0 saline	—	1.0 cc.		0.5 cc.	0.5 c.c	Complete hemolysis.

* May be omitted and 0.5 cc. saline added instead.

2. To each of the first 9 tubes add 0.5 cc. of a 1 to 10 dilution of *normal* human serum previously heated at 55° C. for fifteen minutes.

3. Add 1 cc. of diluted complement (carrying 2 full units) to each of the 10 tubes.

4. To the ninth tube add 0.5 cc. and to the tenth tube 1 cc. saline solution.

5. Mix all tubes and place in a refrigerator at 6° to 10° C. for fifteen to eighteen hours followed by ten minutes in a water-bath at 38° C.

6. Add 0.5 cc. hemolysin (2 units) and 0.5 cc. of the 2 per cent suspension of corpuscles to each tube; mix and place in a water-bath at 38° C. for one hour. Place the tubes in a refrigerator for a few hours and read the results.

7. The *anticomplementary unit is the smallest amount of antigen producing some inhibition of hemolysis;* it is generally 0.5 cc. of 1 to 5 to 1 to 10. The ninth tube is the serum control; the tenth tube is the hemolytic system control and both should show complete hemolysis.

Table 58 shows the ensemble, the results of a titration and method of reading.

Antigenic Titration.—1. In a series of 12 test tubes prepare the following dilutions of antigen starting with the remainder of the 1 to 10 dilution prepared above for the hemolytic and anticomplementary titrations:

0.1 cc. antigen 1 to 10	to 2.9 cc. saline	=	1 to 300
0.1 cc. antigen 1 to 10	to 3.9 cc. saline	=	1 to 400
0.1 cc. antigen 1 to 10	to 4.9 cc. saline	=	1 to 500
1.0 cc. antigen 1 to 300	to 1.0 cc. saline	=	1 to 600
1.0 cc. antigen 1 to 400	to 1.0 cc. saline	=	1 to 800
1.0 cc. antigen 1 to 500	to 1.0 cc. saline	=	1 to 1000
1.0 cc. antigen 1 to 600	to 1.0 cc. saline	=	1 to 1200
1.0 cc. antigen 1 to 800	to 1.0 cc. saline	=	1 to 1600
1.0 cc. antigen 1 to 1000	to 1.0 cc. saline	=	1 to 2000
1.0 cc. antigen 1 to 1200	to 1.0 cc. saline	=	1 to 2400
1.0 cc. antigen 1 to 1600	to 1.0 cc. saline	=	1 to 3200
1.0 cc. antigen 1 to 2000	to 1.0 cc. saline	=	1 to 4000

2. Arrange a series of 14 regulation test tubes and place 0.5 cc. of the above dilutions of antigen into the first 12 tubes, respectively.

3. In each of the first 13 tubes place 0.5 cc. of a 1 to 10 dilution of a mixture of equal parts of four or more freshly collected syphilitic sera yielding strongly positive reactions like 442— — and previously heated at 55° C. for fifteen minutes.

4. In each tube place 1 cc. of diluted complement (carrying 2 full units).

5. To the thirteenth tube add 0.5 cc. and to the fourteenth tube 1 cc. of saline solution.

6. Mix contents of all tubes and place in a refrigerator at 6° to 10° C. for fifteen to eighteen hours.

7. Place tubes in a water-bath at 38° C. for ten minutes and add 0.5 cc. hemolysin (2 units) and 0.5 cc. of the 2 per cent corpuscles

suspension to all tubes; mix and place in a water-bath at 38° C. for one hour. Place tubes in refrigerator for a few hours and read the results.

8. The *antigenic unit is the highest dilution of antigen giving complete inhibition of hemolysis* and is usually 0.5 cc. of 1 to 2400 to 1 to 4000 or three hundred to six hundred times less than the anticomplementary unit. The thirteenth tube is the serum control and the fourteenth tube the hemolytic system control; both should show complete hemolysis.

Table 59 shows the ensemble, the results of a titration and method of reading:

TABLE 59.—ANTIGENIC TITRATION OF ANTIGEN.

Tube	Antigen 0.5 cc.	Heated syphilitic sera 1:10	Complement (2 full units).		Hemolysin (2 units) units).	Corpuscles (2 per cent.)	Water-bath one hour.
1	1:300	0.5 cc.	1.0 cc.	Refrigerator at 6° to 10° C. for fifteen to eighteen hours followed by water-bath for ten minutes.	0.5 cc.	0.5 cc.	Complete inhibition of hemolysis.
2	1:400	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete inhibition of hemolysis.
3	1:500	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete inhibition of hemolysis.
4	1:600	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete inhibition of hemolysis.
5	1:800	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete inhibition of hemolysis.
6	1:1000	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete inhibition of hemolysis.
7	1:1200	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete inhibition of hemolysis.
8	1:1600	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete inhibition of hemolysis.
9	1:2000	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete inhibition of hemolysis.
10	1:2400	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	*Complete inhibition of hemolysis; unit.*
11	1:3200	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Marked inhibition of hemolysis.
12	1:4000	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Slight inhibition of hemolysis.
13	0.5 saline	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	Complete hemolysis.
14	1.0 saline	..	1.0 cc.		0.5 cc.	0.5 cc.	Complete hemolysis.

9. Ten antigenic units are used in conducting the complement-fixation tests. For example, if the unit is 0.5 cc. of 1 to 2400 dilution as shown in Table 59, the dose of 10 units would be contained in 0.5 cc. of 1 to 240 dilution. As a general rule this dose of 10 units is thirty to sixty times less than the anticomplementary unit which largely accounts for the high specificity of the reaction.

THE QUANTITATIVE COMPLEMENT-FIXATION TEST.

1. The tests should be set up in the following order: serum first, followed by antigen, and lastly by complement.

2. For each *serum* arrange 6 regulation test-tubes and place saline solution in the following amounts respectively: 1.2, 0.5, 0.5, 2, 0.5 and 0.5 cc. An automatic pipette is especially useful.

(*a*) Into the first tube place 0.3 cc. serum and mix by drawing up in pipette and expelling one or more times. Transfer 0.5 cc. to Tube No. 6 and 0.5 cc. to Tube No. 2.

(*b*) Mix No. 2 and transfer 0.5 cc. to Tube No. 3.

(*c*) Mix No. 3 and transfer 0.5 cc. to Tube No. 4.

(*d*) Mix No. 4 and transfer 0.5 cc. to Tube No. 5; discard 1.5 cc.

(*e*) Mix No. 5 and discard 0.5 cc.

Each tube now contains 0.5 cc. carrying 0.1, 0.05, 0.025, 0.005, 0.0025 and 0.1 cc. (serum control).

3. For each *spinal fluid* arrange 6 regulation tubes and place 0.5 cc. saline in Nos. 2, 3, 4, 5, and 6.

(*a*) Into the first, second and sixth tubes place 0.5 cc. spinal fluid; mix No. 2 and transfer 0.5 cc. to No. 3; mix No. 3 and transfer 0.5 cc. to tube No. 4; mix No. 4 and transfer 0.5 cc. to tube No. 5. Mix No. 5 and discard 0.5 cc.

(*b*) Each tube now contains 0.5 cc. carrying 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.5 cc. (control).

4. Into the first 5 tubes of each set add 0.5 cc. antigen dilution (carrying 10 antigenic units).

5. After an interval of five to thirty minutes add 1 cc. of complement to each tube (carrying 2 full units).

6. Include the following controls: (*a*) *antigen control* tube carrying 0.5 cc. of the diluted antigen, 1 cc. of the diluted complement and 0.5 cc. of saline solution; (*b*) *hemolytic system control* carrying 1 cc. of the diluted complement and 1 cc. of saline solution; (*c*) *corpuscle control* carrying 2.5 cc. of saline solution; (*d*) *positive* and *negative* serum controls should be included using syphilitic and normal sera, respectively, set up in the various amounts as described above. These controls may be made up with sera carried over in the refrigerator with or without the addition of preservatives as described on page 278 but dried sera have proven more satisfactory in my experience as described on page 281. For use the contents of a tube of positive serum containing the dried residue of 0.3 cc should be dissolved in 1.5 cc. of saline solution and heated at 55° C. for fifteen minutes; 0.5 cc. is placed in Tubes No. 2 and 6 of the quantitative test which leaves 0.5 cc. in the tube as No. 1 of the series; No. 2 is mixed and 0.5 cc. transferred to No. 3, etc., as described above. A normal or negative serum is used in the same manner.

7. Start the preparation of a reading scale as follows (may be omitted after experience in reading the reactions has been gained):

(*a*) Heat 6 cc. of the diluted complement in a water-bath at 55° C. for fifteen minutes.

(*b*) Prepare a solution of hemoglobin by dissolving 2 cc. of the 2 per cent corpuscle suspension in 4 cc. of plain water.

(*c*) Arrange a series of 5 regulation test-tubes numbered 1 to 5 and place in each 0.5 cc. of the diluted antigen and 1 cc. of the heated diluted complement.

(*d*) Add hemoglobin solution to the first 4 tubes as follows: 1.5, 1.13, 0.75 and 0.38 cc.

(*e*) Add 0.24 cc. physiological saline solution to No. 2; 0.5 cc. to No. 3; 0.74 cc. to No. 4 and 1 cc. to No. 5. Corpuscles are added the following day.

8. Mix the contents of all tubes gently but thoroughly and place in a refrigerator at 6° to 10° for fifteen to eighteen hours.

9. Warm the tubes in a water-bath at 38° C. for *ten to fifteen minutes but not longer* and add 0.5 cc. hemolysin (carrying 2 units) to all tubes except the corpuscle control and reading scale; *thoroughly mix* the 2 per cent corpuscle suspension (which has been carried over in a refrigerator from the previous day) and add 0.5 cc. to all tubes except those of the reading scale.

10. Add corpuscle suspension to the tubes of the reading scale as follows: 0.13 cc. to No. 2; 0.25 cc. to No. 3; 0.38 cc. to No. 4 and 0.5 cc. to No. 5.

11. Mix the contents of all tubes gently but thoroughly and place in a water-bath at 38° C. for one hour (the water must reach above the level of the contents in the tubes). Table 60 shows the ensemble.

TABLE 60.—THE QUANTITATIVE COMPLEMENT-FIXATION TEST FOR SYPHILIS.

Tube.	Serum* in 0.5 cc.	Antigen 10 units.		Complement 2 *full* units.		Hemolysin 2 units.	Corpuscles 2 per cent.	
1	0.1 cc.	0.5 cc.	Interval of 10 to 30 minutes at room temperature.	1.0 cc.	Primary incubation in refrigerator at 6° to 10° C. for 15 to 18 hours followed by 10 to 15 minutes at 38° C.	0.5 cc.	0.5 cc.	Secondary incubation in water-bath at 38° C.
2	0.05 cc.	0.5 cc.		1.0 cc.		0.5 cc.	0.5 cc.	
3	0.025 cc.	0.5 cc.		1.0 cc.		0.5 cc.	0.5 cc.	
4	0.005 cc.	0.5 cc.		1.0 cc.		0.5 cc.	0.5 cc.	
5	0.0025 cc.	0.5 cc.		1.0 cc.		0.5 cc.	0.5 cc.	
6	0.1 cc. (control)	None		1.0 cc.		0.5 cc.	0.5 cc.	
7	Antigen control 0.5 cc. saline	0.5 cc.		1.0 cc.		0.5 cc.	0.5 cc.	
8	Hemolytic control 1 cc. saline	None		1.0 cc.		0.5 cc.	0.5 cc.	
9	Corpuscle control 2.5 cc. saline	None		None		None	0.5 cc.	

* Spinal fluid doses: 0.5, 0.25, 0.125, 0.06, 0.03 cc. and 0.5 cc. (control).

12. The reading may be made at once or after placing the tubes in a refrigerator for an hour to permit the partial settling of non-hemolyzed corpuscles. The degree of inhibition hemolysis is then read off and recorded for each tube with the aid of the reading scale as −, + (1), ++ (2), +++ (3),or ++++ (4). All serum controls, antigen and hemolytic controls should show complete hemolysis. As stated in Chapter XXVI more sensitive readings may be made *after sufficient experience has been gained* by reading the reactions ten minutes *after complete hemolysis of the antigen* control providing the serum controls are also completely hemolyzed.

13. Tube No. 1 of the color scale shows a − reaction; Tube No. 2 shows a +; Tube No. 3 shows a ++; Tube No. 4 shows a +++ and Tube No. 5 shows a ++++ reaction.

Experience has shown that the reactions may be interpreted as follows (see Chapter XXVII):

Very strongly positive if there is partial or complete fixation of complement in the first four or all five of the fixation series. Examples: 4431−; 44421; 1442−.

Strongly positive if there is partial or complete fixation of complement in the first three tubes. Examples: 441−−; 421−−; 241−−.

Moderately positive if there is partial or complete fixation of complement in the first two tubes. Examples: 44−−−; 41−−−; 31−−−; 12−−−.

Weakly positive if there is partial or complete fixation of complement only in the first tube. Examples: 4−−−−; 3−−−−; 1−−−−.

Doubtfully positive if ± in the first tube as ± −−−−.

Negative when all tubes show complete hemolysis (−−−−−).

The following reactions with a syphilitic serum and spinal fluid show the method of recording and reporting (Fig. 56):

Quantitative reaction.—Very strongly positive (4421−).	
Serum 0.1	cc. = ++++
Serum 0.05	cc. = ++++
Serum 0.025	cc. = ++
Serum 0.005	cc. = +
Serum 0.0025	cc. = −
Serum 0.1 (control)	cc. = −
Quantitative reaction.—Very strongly positive (44442).	
Spinal fluid 0.5	cc. = ++++
Spinal fluid 0.25	cc. = ++++
Spinal fluid 0.125	cc. = ++++
Spinal fluid 0.0625	cc. = ++++
Spinal fluid 0.03125	cc. = ++
Spinal fluid 0.5 (control)	cc. = −

THE QUALITATIVE COMPLEMENT-FIXATION TEST.

The qualitative test is conducted in the same manner as the quantitative test except that 3 tubes instead of 6 are employed; otherwise the technique is the same and may be described more briefly:

1. For each serum arrange 3 regulation test-tubes and place saline solution as follows: 1.2 cc. in No. 1 and 0.5 cc. in Nos. 2 and 3. Place 0.3 cc. of *serum* into No. 1; mix and transfer 0.5 cc. to Nos. 2 and 3; mix No. 2 and discard 0.5 cc.

2. For each *spinal fluid* arrange 2 regulation test-tubes and place 0.5 cc. in each. The first tube receives antigen; the second does not and is the control.

3. Add 10 units of antigen (0.5 cc. dilution) to the first 2 tubes of each serum and to the first tube of spinal fluid.

4. After waiting five to thirty minutes add 1 cc. of diluted complement (carrying 2 full units) to all tubes of each set.

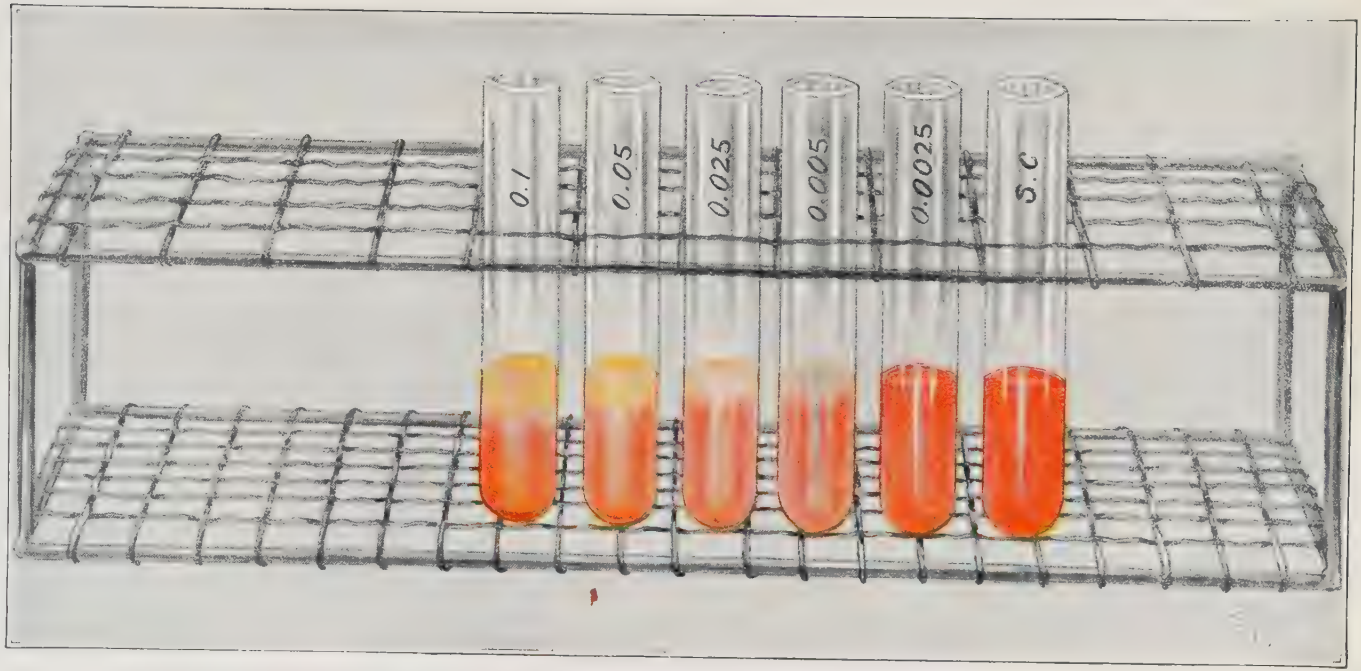

FIG. 56.—A POSITIVE REACTION WITH THE NEW COMPLEMENT-FIXATION TECHNIC.

Shows test-tubes slightly reduced in size, the volume in each and depth of color. The reaction is 4421 (very strongly positive). (*Am. Jour. Syphilis*, 1922, 6, 92.)

5. Set up antigen, hemolytic system and corpuscle controls as described above in the quantitative test; also a syphilitic serum and normal serum for positive and negative controls. Controls may be made up with sera carried over in the refrigerator but dried sera have proven more satisfactory as described on page 281. For use a tube carrying the dried residue of 0.3 cc. of positive serum is dissolved in 1.5 cc. of saline solution and heated at 55° C. for fifteen minutes; 0.5 cc. is transferred to No. 2 and No. 3 which leaves 0.5 cc. as Tube No. 1 of the series; No. 2 is mixed and 0.5 cc. discarded. A normal or negative serum is set up in the same manner.

6. Set up first part of the reading scale as described with the quantitative test (may be omitted).

7. Mix the contents of all tubes gently but thoroughly and place all tubes in a refrigerator at 6° to 10° C. for fifteen to eighteen hours.

8. Warm the tubes in a water-bath at 38° C. for ten to fifteen minutes; add 0.5 cc. hemolysin (2 units) to all tubes except the corpuscle control and reading scale. Gently but thoroughly mix the 2 per cent corpuscle suspension carried over in the refrigerator from the previous day and add 0.5 cc. to all tubes except those of the reading scale.

9. Finish the reading scale by adding corpuscles as described above in the quantitative test.

10. Mix the contents of all tubes gently but thoroughly and incubate in a water-bath at 38° C. for one hour. Table 61 shows the ensemble.

TABLE 61.—THE QUALITATIVE COMPLEMENT-FIXATION TEST FOR SYPHILIS.

Tube.	Serum in 0.5 cc.	Antigen 10 units.	Interval of 10 to 30 minutes at room temperature.	Complement 2 *full* units.	Primary incubation in refrigerator at 6° to 10° C. for 15 to 18 hours followed by 10 to 15 minutes at 38° C.	Hemolysin 2 units.	Corpuscles 2 per cent.	Secondary incubation in water-bath at 38° C.
1	0.1 cc.	0.5 cc.		1.0 cc.		0.5 cc.	0.5 cc.	
2	0.05 cc.	0.5 cc.		1.0 cc.		0.5 cc.	0.5 cc.	
3	0.1 cc. (control)	None		1.0 cc.		0.5 cc.	0.5 cc.	
4	Antigen control 0.5 cc. saline	0.5 cc.		1.0 cc.		0.5 cc.	0.5 cc.	
5	Hemolytic control 1 cc. saline	None		1.0 cc.		0.5 cc.	0.5 cc.	
6	Corpuscle control 2.5 cc. saline	None		None		None	0.5 cc.	

11. Read at once or after the tubes have been placed in a refrigerator for an hour to permit the partial settling of non-hemolyzed corpuscles; read the results with the aid of the scale. All serum, antigen and hemolytic controls should show complete hemolysis; the corpuscle control should show no hemolysis. More sensitive readings may be made after experience has been gained by reading the reactions ten minutes after complete hemolysis of the antigen and serum controls (see page 328).

12. The results are read with the aid of the reading scale as previously described and recorded according to the ++++, +++, ++, + and − method of recording complement-fixation in the first 2 tubes of each set carrying serum or the first tubes of spinal fluid tests:

++++ = strongly positive (100 per cent inhibition of hemolysis).
+++ = moderately positive (about 75 per cent inhibition of hemolysis).
++ = weakly positive (about 50 per cent inhibition of hemolysis).
+ = very weakly positive (about 25 per cent inhibition of hemolysis).
− = negative (complete hemolysis).

In reading the serum reactions the degree of complement-fixation is recorded according to the strongest reaction occurring in *either* of the first 2 tubes. For example, a 13− reaction would be reported as moderately positive because the second tube shows a +++ even though the first shows but +. Additional examples are as follows and illustrate the method:

4,4 = strongly positive
4,3 = strongly positive
3,1 = moderately positive
2,3 = moderately positive
2,1 = weakly positive
1,2 = weakly positive
1,– = very weakly positive
–,1 = very weakly positive
–, = negative

ANALYSIS OF DIFFICULTIES.

Defective Saline Solution.—When trouble is experienced with the hemolytic system when first using these methods it is likely that the saline solution is at fault as discussed with more detail on page 112. If it has been prepared with distilled water I advise trying a saline prepared by dissolving 8.5 gms. of chemically pure sodium chloride in 1000 cc. of ordinary tap water; it is sometimes advantageous to add 0.1 gm. of magnesium sulphate. If, however, trouble is experienced after a saline has been previously used with success it is unlikely to be the cause. I believe that saline prepared of tap water and chemically pure sodium chloride is to be preferred in most localities; compressed tablets of salt should not be used.

Defective Complement.—The complement may be defective in hemolytic activity and this is especially true during the hot summer months when the matter of diet of guinea-pigs may be responsible. As previously stated a mixture of sera from *full grown*, healthy and *previously unused* pigs should be used but I have encountered batches of apparently healthy guinea-pigs whose sera were defective. To be satisfactory the exact unit of complement should not be more than 0.4 cc. of 1 to 30 dilution when titrated as described. If more

than this the serum should not be used since the reactions are likely to be unsatisfactory because such complement is also very likely to be supersensitive to the anticomplementary effects of antigen alone and patients' sera alone.

Defective Hemolysin.—This is probably least likely to be a cause of trouble although usually first suspected and especially if the hemolysin had been previously used with success. The unit of antisheep hemolysin should be at least 0.5 cc. of 1 to 4000 and sera of this and higher strengths are so easy to prepare that it is a mistake to use weaker products. The serum-glycerin mixture deteriorates but very slowly even during the hot summer months and I have many times sent the serum so preserved to all parts of the United States, Australia, China, South America and various parts of Europe with negligible deterioration. If the saline solution and complement are satisfactory a good hemolysin is rarely responsible even when shipped over long distances.

Defective Corpuscles.—When blood is obtained from an abattoir one is almost sure sooner or later to encounter the corpuscles of occasional animals possessing *increased resistance* to serum hemolysis as discussed on page 130. The cause of this phenomenon is unknown; fortunately it is rare. The remedy is to discard the corpuscles and secure a fresh supply of blood.

Anticomplementary Antigen.—Providing no mistakes have occurred in dilution and dosage this is very rarely a cause of trouble. Since 1922 when the new antigen was first employed, none have become anticomplementary in my laboratory over months and years of time. Nor have I been informed of antigens acquiring anticomplementary properties during shipment to all parts of the world. When the antigen control shows incomplete hemolysis it is almost surely due to some component of the hemolytic system and especially the complement.

Anticomplementary Sera.—As discussed in Chapter VII sera and spinal fluids may be found to be anticomplementary as shown by incomplete hemolysis of the serum controls. After experience has been gained some of these reactions may be safely read as discussed in Chapter XXXVIII but as a general rule it is safer and wiser to repeat the tests with fresh serum and especially in the case of those technicians lacking experience in complement-fixation work. It is infinitely better to repeat the tests than to run the slightest chances of error and especially the regrettable and almost unpardonable error of rendering a falsely positive report Sometimes the majority of sera of a day's work show incomplete hemolysis of the serum controls but this trouble is not due to anticomplementary effects on their part but rather to the use of a defective supersensitive complement. Under these conditions the tests must be repeated and

for this reason *the unused portions of all sera should be routinely kept in a refrigerator until the tests are completed in case repetitions are required.*

MODIFIED TECHNIQUE FOR SMALL AMOUNTS OF SERUM AND SPINAL FLUID.

This modification enables one to conduct the quantitative test with but 0.1 cc. of serum instead of the unusal 0.3 cc. and with 0.5 cc. of spinal fluid instead of the usual 1.5 cc. when larger amounts are not obtainable for the routine tests and particularly in the case of children. The technique is as follows:

1. *The hemolysin and complement are not titrated separately for this test but the same dilutions employed as in the routine test described above except that smaller amounts are employed as given below. The same antigen dilution is also used in a smaller dose.* In other words the test is to be used in conjunction with the routine test for the examination of exceptionally small amounts of serum and spinal fluid which are almost surely to be submitted occasionally in routine work.

2. For each *serum* arrange 6 small test tubes and place saline solution: 1.4, 0.5, 0.5, 2 and 0.5 cc. in the first 5 respectively.

3. Place 0.1 cc. heated serum in No. 1; mix, transfer 0.5 cc. to No. 2 and No. 6; mix No. 2 and transfer 0.5 cc. to No. 3; mix, transfer 0.5 cc. to No. 4 and discard 1.5 cc.; mix, transfer 0.5 cc. to No. 5 and discard 0.5 cc. The doses are one-third those of the routine test.

4. In testing *spinal fluid* arrange 6 tubes and place saline solution: 1 cc. in No. 1 and 0.5 cc. in Nos. 2, 3, 4 and 5. Into No. 1 place 0.5 cc. of unheated fluid, mix and transfer 0.5 cc. to Nos. 2 and 6; mix No. 2 and transfer 0.5 cc. to No. 3; mix No. 3 and transfer 0.5 cc. to No. 4; mix No. 4 and transfer 0.5 cc. to No. 5; mix No. 5 and discard 0.5 cc. The doses are one-third those of the routine test.

5. Add 0.2 cc. of antigen dilution to the first 5 tubes of each set.

6. Prepare an *antigen control* with 0.2 cc. antigen and 0.5 cc. of saline solution.

7. Wait ten to thirty minutes and add 0.4 cc. of the complement dilution for the routine tests to all tubes including the antigen control.

8. Prepare a *hemolytic system control* with 0.4 cc. of complement dilution and 0.7 cc. of saline solution.

9. Mix all tubes and place in refrigerator at 6° to 10° C. for fifteen to eighteen hours followed by ten to fifteen minutes in a water-bath at 38° C.

10. Add 0.2 cc. of hemolysin dilution to all tubes.

11. Add 0.2 cc. of the 2 per cent corpuscle suspension to all tubes.

12. Prepare a *corpuscle control* with 0.2 cc. of suspension and 1.3 cc. of saline solution.

13. Mix all tubes and place in a water-bath at 38° C. for one hour. The total volume is 1.5 cc. in all tubes.

14. Read the results at once or after waiting for an hour or two for the partial settling of corpuscles.

15. The readings are made and recorded in exactly the same manner as in the routine quantitative test described.

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CHAPTER XXXII.

TECHNIQUE OF THE KOLMER COMPLEMENT-FIXATION TESTS FOR TUBERCULOSIS, GONORRHEA, TYPHOID FEVER, GLANDERS, CONTAGIOUS ABORTION AND OTHER BACTERIAL DISEASES.

Since the technique of the various bacterial complement-fixation tests is almost exactly the same as for the syphilis reaction, it may be more briefly described. The methods of preparing the antigens are of most importance and those which I employ, based upon the studies described in Chapter XV, are presented in detail. The titration of antigen is exactly the same as in the syphilis reaction except that the dose employed in conducting the main tests is relatively much larger, being from one-third to one-fourth the anticomplementary unit. The primary incubation is the same except that after incubation at 6° to 10° C. for sixteen to eighteen hours, water-bath incubation at 38° C. is for thirty minutes instead of the ten to fifteen minutes recommended for the syphilis test. A substitute primary incubation of two hours at 38° C. in a water-bath may be used and especially in conducting the tuberculosis complement-fixation test.

PREPARATION OF TUBERCULOSIS ANTIGEN.

As stated in Chapter XV, I believe that it is advisable to remove a portion of the lipoids to reduce the degree of cross complement-fixation reactions with syphilis antibody, although prolonged extractions are inadvisable since the acetone-insoluble fraction of these lipoids possesses antigenic properties for tuberculosis antibody.

1. Cultivate human tubercle bacilli in glycerin broth for about four weeks and autoclave the flasks at 10 pounds pressure for twenty minutes to kill the organisms.

2. Filter on several layers of good paper and wash the bacillary residue free of glycerin with sterile water.

3. Transfer the residue to a desiccator and dry over sulphuric acid.

4. Grind in a mortar under a hood for one-half hour and keep in a tightly-stoppered bottle at room temperature.

5. Place 1 gram of powder in a small Erlenmeyer flask fitted with a Liebig's condenser and electric heater as shown on page 377 and boil gently for one hour with 200 cc. of ether. Discard the ether; dry the residue by placing the flask in an incubator, add 200 cc. of acetone and boil for one hour. Discard the acetone, add 200 cc. of absolute ethyl alcohol and boil for one hour. Discard the alcohol.

6. Dry the residue in the flask, add 190 cc. of distilled water and boil for three hours. Add 2 grams sodium chloride to render isotonic and 10 cc. of 5 per cent tricresol or phenol as a preservative. Store in a tightly-stoppered bottle in a refrigerator for at least one week to ripen before titration.

A *second method* may be used omitting the extractions with ether, acetone and alcohol but otherwise exactly as described above. This antigen is sometimes (not always) more antigenic than the first described but likewise much more likely to yield non-specific or cross complement-fixation reactions with syphilis antibody.

PREPARATION OF ANTIGENS OF GONOCOCCI, TYPHOID BACILLI, GLANDERS BACILLI, CONTAGIOUS ABORTION BACILLI, STREPTOCOCCI AND OTHER BACTERIA.

First Method.—This method is especially adapted for preparing antigens of the gonococcus and other organisms difficult of cultivation on a large scale. Both it and the method that follows are based upon the principle of utilizing whole organisms as discussed in Chapter XV.

1. Cultivate the organism on a suitable solid medium and wash off the growths with sufficient sterile distilled water to give a suspension containing approximately 2,000,000,000 per cubic centimeter. Or the organism may be cultivated in a suitable fluid medium, centrifugated and the residue suspended in sterile water to the same concentration.

2. Shake the suspension with glass beads for an hour to break up clumps.

3. Transfer to an Erlenmeyer flask fitted with a Liebig's condenser and electric heater as shown on page 377 and boil gently for two hours. Or the suspension may be boiled in an Arnold sterilizer for the same time making up for any loss in volume with sterile water.

4. Add 1 gram of sodium chloride and 5 cc. of 5 per cent tricresol or phenol to each 100 cc.; stopper tightly and keep in a refrigerator for a week to ripen before titrating.

Second Method.—This method is particularly serviceable for preparing antigens of organisms readily secured in large amounts, as storing in dry powdered form provides a means for keeping indefinitely the base from which antigen may be made up in small amounts as required.

1. Cultivate the organism on a suitable solid medium and remove with a *minimum* amount of sterile saline solution or cultivate in a fluid medium and secure the organisms by centrifugation.

2. Dry the residue in a desiccator over sulphuric acid and grind for one-half hour under a hood.

3. Store the powder in ampoules or in a tightly-stoppered bottle at room temperature.

4. For use place 1 gram in 190 cc. of sterile distilled water and boil with a condenser as shown on page 377 for two hours; or boil in an Arnold sterilizer for the same time making up for any loss in volume by adding saline solution.

5. Add 2 grams of sodium chloride and 10 cc. of 5 per cent phenol or tricresol. Place in a tightly-stoppered bottle in a refrigerator to ripen for a week before titrating.

Smaller amounts may be made up in the same proportions.

TITRATION OF BACTERIAL ANTIGENS.

The technique is exactly the same as described on page 394 for titration of antigen for the syphilis reaction except that the dilutions are slightly different. Table 62 gives directions for preparing the dilutions and it makes no difference whether antigen is added to saline or saline to antigen:

TABLE 62.—DIRECTIONS FOR DILUTING BACTERIAL ANTIGENS FOR TITRATIONS.

2.0 cc. of full strength	plus 2.0 cc. saline	1 to 2
1.0 cc. of full strength	plus 2.0 cc. saline	1 to 3
1.0 cc. of 1 to 2	plus 1.0 cc. saline	1 to 4
1.0 cc. of 1 to 3	plus 1.0 cc. saline	1 to 6
1.0 cc. of 1 to 4	plus 1.0 cc. saline	1 to 8
0.5 cc. of 1 to 2	plus 2.0 cc. saline	1 to 10
1.0 cc. of 1 to 6	plus 1.0 cc. saline	1 to 12
1.0 cc. of 1 to 8	plus 1.0 cc. saline	1 to 16
1.0 cc. of 1 to 10	plus 1.0 cc. saline	1 to 20
0.2 cc. of 1 to 4	plus 1.8 cc. saline	1 to 40
0.2 cc. of 1 to 6	plus 1.8 cc. saline	1 to 60
0.2 cc. of 1 to 8	plus 1.8 cc. saline	1 to 80
0.2 cc. of 1 to 10	plus 1.8 cc. saline	1 to 100
0.2 cc. of 1 to 12	plus 1.8 cc. saline	1 to 120
1.0 cc. of 1 to 80	plus 1.0 cc. saline	1 to 160
1.0 cc. of 1 to 100	plus 1.0 cc. saline	1 to 200
0.5 cc. of 1 to 100	plus 1.0 cc. saline	1 to 300
0.5 cc. of 1 to 200	plus 0.5 cc. saline	1 to 400

Table 63 shows the method of titrating for *hemolytic activity* but since bacterial antigens prepared by the methods given above are rarely hemolytic, this titration may be safely omitted:

TABLE 63.—HEMOLYTIC TITRATION OF BACTERIAL ANTIGEN.

Tube.	Antigen 0.5 cc.	Heated human serum* 1 to 10	Saline.	6° to 10° C. for 15 to 18 hours followed by 30 minutes in water-bath.	Corpuscles 2 per cent.	Water-bath one hour.	Readings.
1	Full strength	0.5 cc.	1.5 cc.		0.5 cc.		Slight hemolysis†
2	1 to 2	0.5 cc.	1.5 cc.		0.5 cc.		No hemolysis
3	1 to 3	0.5 cc.	1.5 cc.		0.5 cc.		No hemolysis
4	1 to 4	0.5 cc.	1.5 cc.		0.5 cc.		No hemolysis
5	1 to 6	0.5 cc.	1.5 cc.		0.5 cc.		No hemolysis
6	1 to 8	0.5 cc.	1.5 cc.		0.5 cc.		No hemolysis

* May be omitted in which case 2 cc. saline is added to each tube instead of 1.5 cc.

† Tubes containing large amounts of antigen should be centrifuged to determine any hemolysis.

Table 64 shows the method of titrating for *anticomplementary activity* and it is the most important of all; it must be conducted at frequent intervals with all antigens:

TABLE 64.—ANTICOMPLEMENTARY TITRATION OF BACTERIAL ANTIGEN.

Tube.	Antigen 0.5 cc.	Heated human serum* 1 to 10	Complement 2 full units.		Hemolysin 2 units.	Corpuscles 2 per cent.		Readings.
1	Full strength	0.5 cc.	1.0 cc.	Refrigerator at 6 to 10° C. for 15 to 18 hours followed by water-bath for 30 minutes; or water-bath only for 2 hours.	0.5 cc.	0.5 cc.	Water bath one hour.	No hemolysis.
2	1 to 2	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		No hemolysis.
3	1 to 3	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		Slight hemolysis.
4	1 to 4	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		Slight hemolysis.
5	1 to 6	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		Marked hemolysis.†
6	1 to 8	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.
7	1 to 10	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.
8	1 to 12	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.
9	1 to 16	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.
10	1 to 20	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.
11	None; 0.5 cc. saline	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.‡
12	None; 1.5 cc. saline	None	None		None	0.5 cc.		No hemolysis.§

* May be omitted and 0.5 cc. of saline added instead.
† Anticomplementary unit being largest amount of antigen giving slight inhibition of hemolysis.
‡ Hemolytic system and serum control.
§ Corpuscle control.

Table 65 shows the method of titrating for *antigenic activity*, which is not essential but always advisable if a suitable positive serum is available:

TABLE 65.—ANTIGENIC TITRATION OF BACTERIAL ANTIGEN.

Tube.	Antigen 0.5 cc.	Heated positive serum* 1 to 5	Complement 2 full units.		Hemolysin 2 units.	Corpuscles 2 per cent		Readings.
1	1 to 10	0.5 cc.	1.0 cc.	Refrigerator at 6 to 10° C. for 15 to 18 hours followed by water-bath for 30 minutes; or water-bath only for 2 hours.	0.5 cc.	0.5 cc.	Water-bath for one hour.	++++
2	1 to 20	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		++++
3	1 to 40	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		++++
4	1 to 60	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		++++†
5	1 to 80	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		+++
6	1 to 100	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		+++
7	1 to 120	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		++
8	1 to 200	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		+
9	1 to 300	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		−
10	1 to 400	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		−
11	0.5 cc. saline	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		−‡
12	1.0 cc. saline	None	1.0 cc.		0.5 cc.	0.5 cc.		−§

* 0.5 cc. of 1 to 5 dilution if a human positive serum heated at 55° C. for fifteen minutes; same if horse or guinea-pig immune sera are used but 0.5 cc. of 1 to 50 if a rabbit immune serum after heating at 60 to 62° C. for thirty minutes.
† The antigenic unit being the smallest amount of antigen giving a ++++ reaction.
‡ Serum control giving complete hemolysis.
§ Hemolytic system control giving complete hemolysis.

THE DOSE OF ANTIGEN TO EMPLOY.

As stated in Chaper XVII bacterial antigens do not appear to give the prezone reactions sometimes observed with the antigens employed in the syphilis reaction and in my experience the most sensitive reactions are always observed with the larger doses of antigen. I employ and recommend *a dose of bacterial antigen equivalent to one-third or one-fourth of the anticomplementary units.* For

example, if the anticomplementary unit of an antigen happens to be 0.5 cc. of 1 to 6 dilution, the dose may be 0.5 cc. of 1 to 18 and this amount would probably carry 2 to 10 antigenic units as determined by the antigenic titration.

The antigenic titration is always advisable if a suitable positive serum is available but if not, the anticomplementary titration alone may be made and the antigen used in an *arbitrary dose* equivalent to one-third or one-fourth of the anticomplementary unit. Even if an antigen happens to be so good that one-third of the anticomplementary unit carries more than 10 antigenic units, I use the one-third to one-fourth dose because the results of the antigenic titration may be particularly favorable by reason of using a positive serum unusually rich in complement-fixing antibody.

In my experience bacterial antigens keep very well in the refrigerator as discussed on page 233; it generally suffices to *titrate but once a month* although one may titrate for anticomplementary activity each time tests are conducted by using the two hour water-bath incubation.

THE QUANTITATIVE AND QUALITATIVE COMPLEMENT-FIXATION TESTS.

The technique of these is exactly as described on pages 397 and 400 except that the primary incubation may be either (*a*) fifteen to eighteen hours in the refrigerator at 6° to 10° C. followed by *one-half* hour in a water-bath at 38° C. or (*b*) two hours in a water-bath. The latter is particularly recommended for the tuberculosis complement-fixation test.

It is advisable to include positive and negative controls and especially the former. The negative controls should be of human sera in the tuberculosis, gonococcus, typhoid and such tests; the positive controls should be of human sera when available but otherwise immune sera of the lower animals may be used as described in Chapter XX; dried sera are particularly useful as stated on page 281.

The readings should be made ten minutes after complete hemolysis of the antigen control in the case of those sera showing complete hemolysis of the serum controls; these give the most sensitive readings. Otherwise the readings should be made *immediately* after the secondary incubation of one hour providing the antigen, serum, hemolytic system and negative serum controls show complete hemolysis.

In conducting the tuberculosis complement-fixation test the Wassermann test should be always conducted at the same time as discussed in Chapter XLIV because syphilis antibody may give a positive reaction with tuberculosis antigen in the absence of tuberculosis. When the Wassermann reaction is strongly positive the tuberculosis test is also quite apt to yield a positive reaction and should be reported upon with great caution. This is not true however, in the case of the gonococcus, typhoid and other bacterial complement-fixation tests.

CHAPTER XXXIII.

TECHNIQUE OF THE KOLMER COMPLEMENT-FIXATION TEST FOR MYCOTIC DISEASES.

The technique of the complement-fixation test for actinomycosis, sporotrichosis, sprue, the ringworms and other mycotic diseases of human beings and the lower animals is exactly the same as described in Chapters XXXI and XXXII for syphilis and the bacterial diseases except in the methods of preparing the respective antigens. These are generally saline preparations and either of the following methods may be employed:

First Method.—The organisms (old cultures preferred) are cultivated on Sabouraud's French proof glucose agar for about four weeks and removed with about 10 cc. of 0.25 per cent phenol or tricresol in distilled water per tube. The suspension is shaken with glass beads in a mechanical shaker for at least four hours and placed in an incubator at 37° C. for at least a week for partial autolysis. The suspension is then briefly centrifuged or filtered through several layers of cheese cloth to remove the heavier particles, rendered isotonic by the addition of 0.1 gram of sodium chloride per 10 cc. and heated in a water-bath at 60° C. for one hour. It should be allowed to ripen in a refrigerator for a few days before titration and use in complement-fixation tests.

Second Method.—This method is practically the same as that described by Lavinder for the preparation of vaccines of ringworm fungi. The cultures are grown in flasks of 100 to 150 cc. of Sabouraud's fluid medium for several weeks. The growths are removed by filtration through sterile paper, washed once or twice with saline solution, transferred to a dish or foil and dried in an incubator or desiccator The dried material is now rubbed into a fine powder and stored in a tightly-stoppered bottle. For use 1 gram is ground with 1 gram of sodium chloride and gradually rubbed with 100 cc. of sterile distilled water. Each cubic centimeter, therefore, contains 0.01 gram in isotonic suspension which is heated at 60° C. for one hour and preserved with 0.25 per cent phenol or tricresol. After standing in a refrigerator for a few days it is titrated for use in complement-fixation tests.

Both antigens keep very well in a refrigerator but should be well shaken before use.

CHAPTER XXXIV.

TECHNIQUE OF THE KOLMER COMPLEMENT FIXATION TESTS FOR ECHINOCOCCUS, TRYPANOSOMIASIS AND OTHER PROTOZOAL AND METAZOAL DISEASES.

Methods for preparing the various antigens are given below as based upon a review of this subject given in Chapter XVI.

The methods for titrating the hemolytic, anticomplementary and antigenic activities are exactly as given on pages 408 to 410 for the titration of bacterial antigens, the hemolytic system and general technique being exactly as described in Chapter XXXI for syphilis.

Each antigen is employed in a dose equivalent to one-third of its anticomplementary unit since the large amounts yield the most sensitive reactions, prezone reactions being quite uncommon. Therefore the anticomplementary unit of each antigen must be known on the basis of preliminary titrations.

In conducting the main tests either the quantitative (page 397) or qualitative (page 400) technique may be employed with a primary incubation of fifteen to eighteen hours in a refrigerator at 6° to 10° C. followed by one-half hour in a water-bath at 38° C. or the water-bath only may be employed for two hours; the former has yielded the more sensitive and satisfactory results in my experience.

In conducting the echinococcus and other tests with human sera, it is advisable (indeed necessary) to conduct a Wassermann test at the same time because all of these antigens are capable of yielding cross complement-fixation reactions with sera containing large amounts of syphilis antibody. Whenever a serum gives a positive Wassermann reaction the results of positive echinococcus or other reactions should be interpreted with due care.

When rabbit immune sera are used for positive controls as described in Chapter XX, the dose should not be more than 0.5 cc. of 1 to 50 dilution (0.01 cc. serum) in order to avoid the non-specific reactions sometimes yielded by normal rabbit serum with these antigens.

The specificity and practical value of complement-fixation reactions in echinococcus disease, bilharziosis and other forms of helminthiasis are discussed in Chapter XLIX.

ANTIGENS OF TRYPANOSOMES.

As a general rule these are prepared of *Trypanosoma equiperdum* for complement-fixation tests with the sera of horses for dourine.

White rats are inoculated[1] and as soon as the tail blood shows

the presence of a heavy infection, antigens are prepared by securing the organisms from the blood by the following or some similar technique:

Method of Reynolds and Schoening.[2]—"Blood of infected rats is collected in a 1 per cent sodium citrate solution in physiological salt solution in order to prevent coagulation. When all the blood has been collected, the solution is filtered through cheese-cloth to remove clots, fibrin, etc., poured into tubes, and centrifugalized for about twenty minutes at 2100 revolutions per minute. This precipitates all the corpuscles and most of the trypanosomes, leaving an upper stratum of blood serum and citrate solution containing some of the organisms. This fluid is drawn off and again centrifugalized in order to recover any of the protozoa which may be present. To the other tubes containing the mass of corpuscles intermixed with and superimposed by trypanosomes is added sufficient distilled water to produce complete hemolysis of the rat erythrocytes, a matter of about twenty minutes, which procedure is facilitated by agitation of the mixture in a flask. This also is centrifugalized but in this instance for about one-half hour, upon the completion of which there is found at the bottom of the tubes a mass of trypanosomes with an admixture of stroma of the hemolyzed red cells, which latter, in quantity, has been found to be negligible. After discarding the supernatant fluid (hemoglobin-stained water) physiological salt solution is added and the material vigorously shaken until the mass of trypanosomes is disintegrated and evenly distributed throughout the solution. Centrifuging is again resorted to with similar results, the washed mass of trypanosomes being packed at the bottom of the tubes. The salt solution is poured off and an amount of preserving fluid (physiological salt solution and glycerin āā) equal to about twice the amount of trypanosomes added; the mixture is then agitated until a uniform suspension is acquired, when it is stored at a low temperature until used."

Antigen may be also prepared by infecting dogs with *Trypanosoma brucei* and securing the parasites from the blood for complement-fixation tests in dourine of horses as described by Moller, Elchhorn and Buck; also by extracting the spleens of rats succumbing to infections with *Trypanosoma equiperdum* or *Trypanosoma brucei*. Both methods are described elsewhere.[3]

ANTIGENS OF HELMINTHS.

Echinococcus Antigen.—The fluid from echinococcus cysts has been usually employed preserved with 0.5 per cent phenol in a refrigerator. It would appear however, that the scolices contain most of the antigenic principles and a better antigen may be prepared by grinding up the moist scolices with fine sand in a mortar and adding 9 volumes of the clear cyst fluid or saline solution to

give an approximate 10 per cent extract of the scolices. Phenol or tricresol should be added to 0.25 per cent and the mixture extracted in an incubator at 37° C. for four days, filtered and stored in a refrigerator.

Fairley[4] recommends an alcoholic extract prepared by grinding the scolices with fine sand, adding 9 volumes of absolute ethyl alcohol to give a 10 per cent extract and placing the mixture in an incubator for two days when it is filtered and stored for use.

Bilharziosis Antigens.—In the serum diagnosis of liver fluke infestations with *Schistosomum japonicum*, *S. mansoni* and *S. hematobium*, antigens may be prepared by extracting the finely ground adult bilharzia worms with saline solution or absolute ethyl alcohol as described above in the preparation of echinococcus antigen. Fairley[5] however, recommends the following method for the preparation of antigen in the routine serum diagnosis of bilharziosis:

"Snails from naturally infected sources (pools, sweet water canals, etc.) are collected and placed over-night in test-tubes containing about 10 cc. of tap water. Next morning they are examined with a hand lens and those showing bilharzia cercariæ are set aside and later dissected, the digestive gland or liver being removed, teased out with needles and placed in absolute alcohol, 1 cc. being used for each liver. The mixture is placed for twenty-four hours at 37° C. (a longer period is not necessary) and well shaken by hand or in an electric shaker for three periods of twenty minutes each. The extract is then run through filter paper and this filtrate may be stored and used for antigen as required."

Fairley has also prepared efficient antigens with dried powdered cercarial livers extracted with absolute alcohol.

Intestinal Helminth Antigens.—Antigens of *Tænia saginata*, *Ascaris lumbricoides*, etc., may be prepared by extracting the moist finely ground parasites with 9 volumes of absolute ethyl alcohol for two to four days at 37° C. Or the parasites may be finely ground, dried, pulverized and 1 part of powder extracted in the same manner. Saline extracts may be prepared of the finely divided moist or dried parasites by extracting with 9 volumes of saline containing 0.25 per cent phenol or tricresol at 37° C. for four days and filtering. The alcoholic extracts are superior.

REFERENCES.

1. For technique see page 207 of Kolmer's Principles and Practice of Chemotherapy with Special Reference to the Treatment of Syphilis, 1926, W. B. Saunders Company, Philadelphia.

2. Reynolds and Schoening: Jour. Agricultural Research, 1918, **14**, 573.

3. Kolmer's Infection, Immunity and Biologic Therapy, W. B. Saunders Company, third edition. 1923, p. 549.

4. Fairley: Med. Jour. Australia, 1922, **1**, 341.

5. Fairley: Jour. Path. and Bacteriol., 1925, **28**, 591.

CHAPTER XXXV.

TECHNIQUE OF THE KOLMER COMPLEMENT-FIXATION TESTS EMPLOYING RABBIT, DOG AND MULE SERA.

As stated in Chapter XXVIII the sera of some of the lower animals and notably of the rabbit, dog and mule sometimes yield non-specific complement-fixation and precipitation reactions with the various antigens employed in the syphilis reactions. With various bacterial antigens the degree of fixation is even greater. *Therefore in conducting complement-fixation reactions with the sera of these animals the technique must be modified to avoid the possibility of these non-specific reactions and yet sufficiently sensitive for the detection of specific antibody.* These ends are met by heating the sera at 62° C. instead of at 55° to 56° C. and by using more complement (first method) or smaller doses of serum (second method).

First Method. The *technique is exactly the same as described in Chapter XXXI for the testing of human sera* except:

1. The natural antisheep hemolysins are not removed from the sera.

2. The sera are heated in a water-bath at 62° C. for thirty minutes.

3. The doses in *syphilis* tests are the same as employed in testing human sera or they may be 0.1, 0.05, 0.025, 0.0125, 0.006 and 0.1 cc. (control) in the quantitative test. But in *bacterial* complement-fixation tests smaller amounts of serum should be used as:

0.025 cc. (0.5 cc. of 1 to 20)
0.012 cc. (0.5 cc. of 1 to 40)
0.006 cc. (0.5 cc. of 1 to 80)
0.003 cc. (0.5 cc. of 1 to 160), etc.
0.025 cc. (0.5 cc. of 1 to 20) in the serum control.

The first two doses of either series are used in the qualitative test, that is, 0.1, 0.05 and 0.1 cc. (control) with cholesterolized and lecithinized alcoholic extract of beef heart antigen in syphilis tests or 0.025, 0.012 and 0.025 cc. (control) in bacterial complement-fixation tests employing the antigen in a dose equivalent to *one-fourth* of its anticomplementary unit.

4. *Four full units of complement* are used instead of 2 and so diluted that this dose is contained in 1 cc. Example:

Exact unit = 0.25 cc. of 1 to 30 dilution
Full unit = 0.30 cc. of 1 to 30 dilution
4 full units = 1.2 cc. of 1 to 30 dilution

To calculate the dilution to use so that 1 cc. contains the dose divide 30 by the dose:

$$\frac{30}{1.2} = 25 \text{ or dilution 1 to 25 in dose of 1 cc.}$$

Second Method.—The technique is exactly the same as described in Chapter XXXI for testing human sera except:

1. The natural antisheep hemolysins are not removed from the sera.
2. The sera are heated in a water-bath at 62° C. for thirty minutes.
3. The doses of serum in quantitative *syphilis* tests with the usual 10 units of antigen are:

0.025 cc. (0.5 cc. of 1 to 20)
0.0125 cc. (0.5 cc. of 1 to 40)
0.006 cc. (0.5 cc. of 1 to 80)
0.003 cc. (0.5 cc. of 1 to 160)
0.0015 cc. (0.5 cc. of 1 to 320)
0.025 cc. (0.5 cc. of 1 to 20) control

The doses in quantitative *bacterial* tests with one-fourth of the anticomplementary unit of antigen are:

0.0125 cc. (0.5 cc. of 1 to 40)
0.006 cc. (0.5 cc. of 1 to 80)
0.003 cc. (0.5 cc. of 1 to 160)
0.0015 cc. (0.5 cc. of 1 to 320)
0.0008 cc. (0.5 cc. of 1 to 640)
0.0125 cc. (0.5 cc. of 1 to 40) control

In qualitative tests the first two doses of either series are employed along with the larger amount in the third tube or serum control.

Heating sera at 62° C. for thirty minutes does not destroy enough antibody in syphilitic rabbits or in rabbits and dogs immunized to various antigens to interfere with the sensitiveness of the reactions; nor does this degree of heating of mule sera interfere with the sensitiveness of the glanders complement-fixation test for which they are usually submitted.

CHAPTER XXXVI.

TECHNIQUE OF THE KOLMER COMPLEMENT-FIXATION TEST FOR THE IDENTIFICATION OF SERA AND BLOOD STAINS.

The principles of the complement-fixation reaction for the medico-legal identification and differentiation of blood stains are discussed in Chapter XXIX and it has proven more sensitive and decisive in my experience than the precipitin reaction for this purpose. The usual order is reversed in that a known antibody serum is used for the detection and identification of an unknown antigen. The technique of the test is described in this chapter while the specificity, sensitiveness and practical applications are discussed in Chapter L.

PREPARATION OF IMMUNE SERA.

In medico-legal work the question usually raised is whether or not a stain or other material is of human blood. An extract is prepared of the material as described below and *if chemical tests show that it is composed of blood*, it is employed as antigen in the complement-fixation tests employing an immune serum known to contain antihuman blood antibody. As a general rule other antisera are also used in duplicate tests and particularly those prepared by immunizing rabbits or guinea-pigs with the bloods of domestic animals like those of the chicken, dog, cat, hog, rabbit, horse, cow and sheep for the identification of any specimen of blood other than of human origin.

It is essential to use immune sera containing large amounts of antibody in order to secure specific and reliable results; this is especially true in medico-legal tests involving the charge of murder and the unit should not be less than 0.5 cc. of 1 to 200 of serum when titrated as described below.

Large, healthy and previously unused rabbits are usually chosen and any one of several methods of immunization may be employed. In my experience *intravenous injections of defibrinated blood have generally yielded better immune sera than injections of serum.*

One of the best methods is the daily intravenous injection of 0.1 cc. of sterile defibrinated blood or serum diluted with 0.9 cc. of sterile saline solution for 21 to 28 injections. By exercising due care with the ear veins no difficulties are encountered, the mortality is low and the yield of antibody usually quite good.

A second method is to give 0.5 cc. of sterile defibrinated blood or serum diluted with 4.5 cc. of sterile saline solution by intravenous injection every five to seven days for 6 to 8 injections.

One week after the last injection by either method some blood is removed from the ear of each animal and a titration made of the antibody content as described below. If the unit is 0.5 cc. of 1 to 200 or higher (preferably 1 to 500 to 1 to 1000) the animal is bled, the serum separated and preserved with an equal part of chemically pure glycerin.

Rabbits vary however, in antibody production and it is always well to immunize two or three at a time with each antigen. This variation is shown in the following results of one set of immunizations by the first method in which the units of complement-fixing antibody were as follows:

Antihuman No. 1	0.5 cc. of 1 to 3000
Antihuman No. 2	0.5 cc. of 1 to 1600
Antihuman No. 3	0.5 cc. of 1 to 200
Antihuman No. 4	0.5 cc. of 1 to 400
Antisheep No. 1	0.5 cc. of 1 to 12,000
Antisheep No. 2	0.5 cc. of 1 to 800
Anticow No. 1	0.5 cc. of 1 to 500
Anticow No. 2	0.5 cc. of 1 to 800
Antidog No. 1	0.5 cc. of 1 to 150
Antidog No. 2	0.5 cc. of 1 to 100
Antihorse No. 1	0.5 cc. of 1 to 500
Antihorse No. 2	0.5 cc. of 1 to 600
Antihog No. 1	0.5 cc. of 1 to 800
Antihog No. 2	0.5 cc. of 1 to 400
Antichicken No. 1	0.5 cc. of 1 to 4000
Antichicken No. 2	0.5 cc. of 1 to 1500
Anticat No. 1	0.5 cc. of 1 to 200
Anticat No. 2	0.5 cc. of 1 to 500

Antirabbit sera are prepared by immunizing guinea-pigs or goats with rabbit blood or serum. Large, healthy and previously unused guinea-pigs may be likewise used for the preparation of other antisera as stated in Chapter XX, although the antibody output is not nearly as high as with rabbits. Their sera have one advantage however, in that they do not yield non-specific reactions as may rabbit sera, although this may be obviated in the latter by using sera in a unit not larger than 0.5 cc. of 1 to 200 dilution (a dose of 2 units being 0.5 cc. of 1 to 100 or less than the non-specific fixing dose of normal rabbit serum).

Guinea-pigs are immunized by an intraperitoneal injection of 0.5 cc. of sterile defibrinated blood or serum diluted with 4.5 cc. of sterile saline solution every five to seven days for 10 to 12 injections. In one set of immunizations the units were as follows:

Antihuman No. 1	0.5 cc. of 1 to 10
Antihuman No. 2	0.5 cc. of 1 to 80
Antihuman No. 3	0.5 cc. of 1 to 40
Antisheep	0.5 cc. of 1 to 30
Anticow No. 1	0.5 cc. of 1 to 20
Anticow No. 2	0.5 cc. of 1 to 5

The injection of rabbits or guinea-pigs with defibrinated sheep blood always produces antisheep hemolysin as well as complement-fixing antibody; injections of sheep serum produce much less hemolysin. But if the complement-fixation tests are conducted with an antisheep system as described below it is advisable to remove the immune hemolysins because their presence constitutes a great excess reducing the sensitiveness of the reactions. This is accomplished by mixing 0.1 cc. of washed sheep corpuscle sediment to each cubic centimeter of serum, placing the mixture in a refrigerator for one hour and removing the sensitized corpuscles by centrifuging, the supernatant serum being preserved as described. Otherwise plain antisheep serum could be tested with an antiox or antihuman hemolytic system in which case the presence of antisheep hemolysin would have no influence.

The method of titration is given below and the sera will keep in a refrigerator practically unchanged after the initial drop in antibody, for months and even one or more years.

PREPARATION OF EXTRACTS (ANTIGENS.)

If the material for testing is a stain on a fabric like a towel, handkerchief, undergarment, etc., a portion is carefully cut away with *clean* scissors and extracted in sterile phenolized saline solution prepared by diluting 1 cc. of pure phenol up to 400 cc. with saline (0.25 per cent and sufficient for preventing bacterial growths). If it appears to be an unwashed stain of blood a piece or aggregate of pieces totalling about 2 inches square may be extracted in 50 cc. of phenolized saline solution. If the stain has been partially washed out, correspondingly larger pieces should be extracted or smaller amounts of phenolized saline employed. Sometimes it is advisable to tear portions into single threads which may show incrustations, especially if the fabric is of a heavy kind. Spots may be likewise scraped from wood, metal and plastered walls with *clean* scalpels and collected in containers or placed at once in phenolized saline solution. As a general rule each 0.1 gram of material should be extracted in about 10 cc. of sterile solution to obtain approximately 1 to 100 extracts. One part of clot should be extracted in 100 parts of sterile phenolized water to give complete hemolysis and later rendered isotonic by adding 1 gram of sodium chloride to each 100 cc. *In medico-legal work it is imperative to exercise every precaution against confusion and the source of every specimen along with other essential data should be carefully recorded.*

It is well to prepare *control extracts* at the same time and in the same manner by extracting similar pieces of *unstained* fabric or other material if available, which is usually the case.

The extracts should be placed in an incubator for twenty-four

hours or longer for the solution of flinty particles from spots on metal or wood. Heating the extracts at about 40° C. for an hour or two sometimes hastens solution. Sometimes it is necessary to promote solution by extracting with 0.5 per cent solutions of sodium hydroxide in saline solution and later neutralizing with decinormal hydrochloric acid with phenolphthalein indicator.

Small portions of blood-soaked plaster, earth, hay, straw, grass, leaves, etc., may be extracted with phenolized saline solution but in the case of leather, wood, bark and earth the reaction should be taken and the extracts brought to the neutral point.

The extracts should be filtered through paper if necessary although in most instances they can be used unfiltered since they do not require the crystal clearness essential for the precipitation test.

Each extract should now be tested chemically for blood by the phenolphthalein, benzidine or some other reliable method. If these or tests applied directly to the material are negative for blood it is hardly advisable to go on with the complement-fixation tests.

If the material for testing happens to be a *serum* a portion is diluted to 1 to 100 with either plain or phenolized saline solution. Fresh sera do not require preliminary heating but older sera should be first heated at 55° C. for thirty minutes to remove antilysins.

TECHNIQUE OF COMPLEMENT-FIXATION TESTS.

The general technique and hemolytic system are exactly the same as described in Chapter XXXI for the syphilis complement-fixation reactions. But only those possessing considerable experience should attempt conducting these tests and especially if medico-legal issues are involved.

Anticomplementary Titration of Extract Antigens.—The anticomplementary activity of each extract must be determined in exactly the same manner as described on page 395 for the anticomplementary titration of antigens for the syphilis complement-fixation test, the doses and arrangement being shown in Table 66. When extracts are kept in a refrigerator the anticomplementary titers remain quite constant over long periods of time. *In the complement-fixation tests each extract is used in variable amounts ranging from one-half to one-twelfth of its anticomplementary unit. If the undiluted extract (first tube) happens to be without anticomplementary activity the doses are arbitrarily 0.5 cc. of undiluted, 1 to 2, 1 to 3, 1 to 4, 1 to 6 and 1 to 8 of the stock solution.*

In the case of antigens prepared of fresh sera in dilutions of 1 to 100, anticomplementary titrations are not usually required since they may be employed in an arbitrary dose of 0.5 cc. of the stock and 1 to 2, 1 to 3, 1 to 4, and 1 to 8 dilutions of it. Older serum antigens should be titrated since they may be anticomplementary and

used in one-half, one-third, one-fourth, one-sixth, one-eighth and one-twelfth of the anticomplementary unit.

TABLE 66.—THE ANTICOMPLEMENTARY TITRATION OF A BLOOD STAIN EXTRACT.

Tube.	Extract 0.5 cc.	Complement 2 full units.	Saline.		Hemolysin 2 units.	Corpuscles 2 per cent.		Readings.
1	Undiluted	1.0 cc.	0.5 cc.	Refrigerator at 6 to 10° C. for 15 to 18 hours followed by water-bath for 10 to 15 minutes.	0.5 cc.	0.5 cc.	Water-bath one hour.	Incomplete hemolysis (unit).*
2	1 to 2	1.0 cc.	0.5 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.
3	1 to 3	1.0 cc.	0.5 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.
4	1 to 4	1.0 cc.	0.5 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.
5	1 to 6	1.0 cc.	0.5 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.
6	1 to 8	1.0 cc.	0.5 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.
7	1 to 12	1.0 cc.	0.5 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.
8	1 to 16	1.0 cc.	0.5 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.
9	—	1.0 cc.	1.0 cc.		0.5 cc.	0.5 cc.		Complete hemolysis (control).
10	—	—	2.5 cc.		—	0.5 cc.		No hemolysis (control).

* Anticomplementary unit 0.5 cc. of undiluted extract. The doses for the complement-fixation tests would be 0.5 cc. of 1 to 2, 1 to 4, 1 to 6, 1 to 8, 1 to 12, and 1 to 16. If this tube showed complete hemolysis (absence of anticomplementary activity) the doses would be 0.5 cc. undiluted, 1 to 2, 1 to 3, 1 to 4, 1 to 6, and 1 to 8 dilutions.

A titration for hemolytic activity is not required.

Titration of Immune Serum.—Each immune serum is titrated with its antigen to determine the smallest fixing dose. An antihuman serum, for example, is titrated with an antigen of human blood; an antidog serum would be titrated with an antigen of dog blood, etc.

1. Dilute 0.1 cc. of serum with 0.9 cc. of saline and heat at 62° C. for thirty minutes; add 19 cc. of saline solution (1 to 200). If the serum is preserved with an equal part of glycerin dilute 0.2 cc. with 0.8 cc. of saline, heat and add 19 cc. of saline (1 to 200).

2. In a series of 10 test-tubes place 0.5 cc. of increasing dilutions shown in Table 67.

TABLE 67.—TITRATION OF ANTISERUM.

Tube.	Antiserum 0.5 cc.	Blood antigen.*	Complement 2 full units.		Hemolysin 2 units.	Corpuscles 2 per cent.		Readings.
1	1 to 200	0.5 cc.	1.0 cc.	Refrigerator at 6° to 10° C. for 15 to 18 hours followed by water-bath for 10 to 15 minutes.	0.5 cc.	0.5 cc.	Water-bath one hour.	++ (prezone).
2	1 to 300	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		++++
3	1 to 400	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		++++
4	1 to 600	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		++++
5	1 to 800	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		++++
6	1 to 1200	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		++++ (unit†)
7	1 to 1600	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		+++
8	1 to 2400	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		++
9	1 to 3200	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		+
10	1 to 4800	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		+
11	0.5 cc. saline	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		− (antigen control).
12	1.0 cc. saline	—	1.0 cc.		0.5 cc.	0.5 cc.		− (hemolytic control).

* Dose one-half of anticomplementary unit; usually 0.5 cc. of 1 to 3 dilution of stock 1 to 100 solution.

† Highest dilution or smallest amount of serum giving ++++ reaction and therefore the unit. In the main tests 10 units or 0.5 cc. of 1 to 120 dilution would be used. If all of the first 10 tubes show ++++ reactions with negative controls, higher dilutions of immune sera would require testing since the unit would be less than 0.5 cc. of 1 to 4800.

3. Prepare an antigen of homologous blood by diluting 1 cc. of defibrinated blood in 100 cc. of sterile water and after complete hemolysis add 1 gram of sodium chloride. Or dissolve 0.2 gram of pulverized dried blood in 20 cc. of sterile saline solution. Titrate for anticomplementary activity and use in dose of one-half the unit although these antigens are almost invariably without anticomplementary activity and may be used in a dose of 0.5 cc. of 1 to 2 dilution of the stock 1 to 100 solution.

4. Complete the test as shown in Table 67. *The smallest amount of immune serum giving a ++++ reaction is the unit* and the bottle or vial may be labelled accordingly. If the serum is titrated one or two weeks after ripening in a refrigerator, the unit remains quite constant over months of time and the serum may be used without retitration. *But in medico-legal cases it is always advisable to retitrate* before setting up the complement-fixation tests. *The amount employed in the complement-fixation tests is 2 to 10 units in 0.5 cc. providing this amount does not exceed 0.5 cc. of a 1 to 100 dilution.* If the unit were just 0.5 cc. of 1 to 200 the dose would be 2 units or 0.5 cc. of 1 to 100. If the unit is less than 0.5 cc. of 1 to 200 the serum should not be used. The following examples illustrate the method:

Unit 0.5 cc. of 1 to 200	dose 0.5 cc. of 1 to 100 (2 units)
Unit 0.5 cc. of 1 to 300	dose 0.5 cc. of 1 to 100 (3 units)
Unit 0.5 cc. of 1 to 400	dose 0.5 cc. of 1 to 100 (4 units)
Unit 0.5 cc. of 1 to 800	dose 0.5 cc. of 1 to 100 (8 units)
Unit 0.5 cc. of 1 to 1200	dose 0.5 cc. of 1 to 120 (10 units)
Unit 0.5 cc. of 1 to 1600	dose 0.5 cc. of 1 to 160 (10 units) etc.

The Complement-fixation Test.—As a general rule human blood is always suspected in medico-legal cases and it generally suffices to show whether or not this is the case without making further effort toward identification if the material happens not to be of human origin. But in all tests it is well to include one or two other immune sera along with their homologous antigens as controls and the general arrangement is shown in Table 68 from the protocols of one of my medico-legal cases involving the charge of murder.

1. The unknown extract is used in six different doses. If the stock solution is not anticomplementary when titrated as shown in Table 66 the doses are 0.5 cc. of stock and 0.5 cc. of 1 to 2, 1 to 3, 1 to 4, 1 to 6 and 1 to 8 dilutions of it (Tubes 1, 2, 3, 4, 5 and 6).

2. If control extracts are included they may be prepared by dissolving 0.5 cc. of defibrinated blood in 50 cc. of sterile water and adding 0.5 gram of sodium chloride . As a general rule they do not require titration and may be used in a single dose of 0.5 cc. of 1 to 2 dilutions as shown in Tubes 10, 11, 12, 16, 17 and 18 of Table 68.

3. The hemolysin is titrated as described on page 392.

4. The complement is titrated in the presence of the *largest amount of each blood extract employed* and used in a dose of 2 full units. The general arrangement is the same as shown on page 393 except that the dose of antigen is usually 0.5 cc. of undiluted or 1 to 2 dilution of stock solution of each extract.

TABLE 68.—THE COMPLEMENT-FIXATION TEST FOR THE IDENTIFICATION OF A BLOOD STAIN.

Tube.	Extracts 0.5 cc. of:	Heated antiserum 0.5 cc. carrying 2 to 10 units.	Complement (2 full units).		Hemolysin (2 units)	Corpuscles (2 per cent.)	Water-bath one hour. results.
1	Unknown stock solution*	Antihuman	1.0 cc.	Refrigerator incubation at 6° to 10° C. for 15 to 18 hours followed by 10 to 15 minutes in a water-bath at 38° C.	0.5 cc.	0.5 cc.	++++‡
2	Unknown 1 to 2 of stock	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	++++
3	Unknown 1 to 3 of stock	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	++++
4	Unknown 1 to 4 of stock	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	++++
5	Unknown 1 to 6 of stock	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	+++
6	Unknown 1 to 8 of stock	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	++
7	Unknown stock solution*	0.5 cc. saline	1.0 cc.		0.5 cc.	0.5 cc.	− (antigen control)
8	Unknown 1 to 2 of stock	Antidog	1.0 cc.		0.5 cc.	0.5 cc.	− (specificity control)
9	Unknown 1 to 2 of stock	Antiox	1.0 cc.		0.5 cc.	0.5 cc.	− (specificity control)
10	Human blood 1 to 2 of stock†	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	++++ (positive control)
11	Dog blood 1 to 2 of stock†	Antidog	1.0 cc.		0.5 cc.	0.5 cc.	++++ (control)
12	Beef blood 1 to 2 of stock†	Antiox	1.0 cc.		0.5 cc.	0.5 cc.	++++ (control)
13	0.5 cc. saline	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	− (serum control)
14	0.5 cc. saline	Antidog	1.0 cc.		0.5 cc.	0.5 cc.	− (serum control)
15	0.5 cc. saline	Antiox	1.0 cc.		0.5 cc.	0.5 cc.	− (serum control)
16	Human blood 1 to 2 of stock*	0.5 cc. saline	1.0 cc.		0.5 cc.	0.5 cc.	− (antigen control)
17	Dog blood 1 to 2 of stock*	0.5 cc. saline	1.0 cc.		0.5 cc.	0.5 cc.	− (antigen control)
18	Beef blood 1 to 2 of stock*	0.5 cc. saline	1.0 cc.		0.5 cc.	0.5 cc.	− (antigen control)
19	1.0 cc. saline	—	1.0 cc.		0.5 cc.	0.5 cc.	− (system control)
20	2.5 cc. saline	—	—		—	0.5 cc.	No hemolysis (corpuscle control)

* Not anticomplementary in 0.5 cc. of stock solutions but each extract is controlled in the maximum amounts employed in tubes 7, 16, 17 and 18.

† Prepared by dissolving 0.5 cc. of defibrinated blood in 50 cc. of water and adding 0.5 gm. of sodium chloride.

‡ Strongly positive; − = complete hemolysis (negative), etc.

5. The antihuman and other antisera are heated at 62° C. for thirty minutes and used in a dose of 2 to 10 units providing this dose is not more than 0.5 cc. of 1 to 100 of serum. If the unit happens to be 0.5 cc. of 1 to 200, 2 units or 0.5 cc. of 1 to 100 may be used. But if the unit is less than 0.5 cc. of 1 to 200 the serum should not be used.

6. When extracts and antisera are mixed there may be an interval of ten minutes before the addition of complement although this is not essential.

7. Each extract is controlled for anticomplementary activity and each should show complete hemolysis, as shown in Tubes 7, 16, 17 and 18.

8. Each antiserum should be controlled for anticomplementary activity and give complete hemolysis, as shown in Tubes 13, 14 and 15.

9. A positive control employing an extract of human blood and antihuman serum should be included and show a strongly positive reaction, as in Tube 10.

10. Likewise controls on each known extract set up with homologous sera as shown in Tubes 11 and 12 and giving strongly positive reactions.

11. A hemolytic system control should be included and give complete hemolysis (Tube 19).

12. A corpuscle control should show no hemolysis (Tube 20).

CHAPTER XXXVII.

TECHNIQUE OF THE KOLMER COMPLEMENT-FIXATION TESTS FOR THE IDENTIFICATION OF MEAT AND MILK ADULTERATION AND SEMINAL STAINS.

As stated in Chapter LII the complement-fixation test has proven superior to the precipitin test for the detection of meat adulteration as, for example, the presence of dog, cat or horse flesh in sausages.

The complement-fixation test is also applicable for the identification of milks although it is lacking in sufficient specificity for the detection of goat's milk added to cow's milk, as discussed in Chapter LIII. The same is true of the precipitin test.

The complement-fixation test may be also applied for the detection of seminal stains although, unfortunately, vaginal secretions free of spermatozoa may give cross reactions. But for the identification of seminal fluids from different species it is highly specific.

THE COMPLEMENT-FIXATION TEST FOR THE DETECTION OF MEAT ADULTERATION.

Preparation of Antigen.—About 50 grams of flesh are removed from the deeper parts of the specimen by means of a sterile knife, and through a fresh opening, as this portion has been least exposed to the methods of preservation, especially at the high temperatures to which meat may have been exposed. It should contain as little fat as possible. It is then *finely minced* and placed in a sterile Erlenmeyer flask with 200 cc. of saline solution and 1 cc. of phenol added (0.5 per cent). The flask is then tightly stoppered, incubated at 37° C. for four days with brief shaking each day, filtered through paper and stored in a refrigerator.

Salted meat should be first minced and extracted with sterile water for fifteen minutes, repeated two or three times, before extraction with phenolized saline:

Sausage and fatty meats should be first extracted with 200 cc. of equal parts of ether and chloroform for twenty-four hours at room temperature before extraction with phenolized saline solution.

The finished extracts should contain enough protein to give a foam upon shaking and a positive heat and acetic acid reaction.

Preparation of Antisera.—For the examination of sausages antihog, antisheep, antibeef, antidog, anticat and antihorse sera should be available.

These are best prepared by the immunization of large, healthy and

previously unused rabbits, several animals being immunized with each antigen.

To prepare antidog serum one may immunize with an extract of dog flesh prepared as described above, with dog serum or a mixture of the two. In my experience best results have been observed with a mixture prepared with 3 parts of meat extract and 1 part of serum. A mixture of this kind keeps quite well in a refrigerator and rabbits may receive intravenous injections at weekly intervals of the following amounts: 1, 2, 4, 5, 6, 8 and 10 cc. If intraperitoneal injections are given the doses should be slightly larger: 2, 4, 5, 8, 10, 10, 15 and 15 cc.

Antisera for hog, sheep, beef, cat, horse and the flesh of other animals may be prepared in the same manner.

Since cooking alters the specificity of the antigens it is advisable to prepare a second set of immune sera with heated flesh and sera, as these antisera will detect the presence of cooked meats in sausages, etc. These are prepared as described above except that the comminuted flesh is first boiled for fifteen minutes, finely minced and extracted with phenolized saline. Serum is diluted 1 to 10, heated at 70° C. for one hour and 1 part added to 3 parts of the extract. The doses are the same as given above.

One week after the last dose, blood should be removed from the ear of each animal and the serum titrated as described below. If strongly positive reactions occur with 0.5 cc. of 1 to 200 or higher dilutions of serum the antibody content is sufficient and the animal may be bled; otherwise 2 or 3 more injections should be given. The serum should be preserved with an equal part of chemically pure glycerin, carefully labelled and kept in a refrigerator. Potent antisera will keep for months and even years with surprisingly little deterioration after the initial drop of antibody occurring during the first week or two after bleeding.

Table 69.—The Anticomplementary Titration of a Meat Extract.

Tube.	Extract 0.5 cc.	Complement 2 full units.	Saline.		Hemolysin 2 units.	Corpuscles 2 per cent.		Readings.
1	Undiluted	1.0 cc.	0.5 cc.	Refrigerator at 6° to 10°C. for 15 to 18 hours followed by water-bath for 10 to 15 minutes.	0.5 cc.	0.5 cc.	Water-bath one hour.	Incomplete hemolysis.
2	1 to 2	1.0 cc.	0.5 cc.		0.5 cc.	0.5 cc.		Incomplete hemolysis.
3	1 to 3	1.0 cc.	0.5 cc.		0.5 cc.	0.5 cc.		Incomplete hemolysis.
4	1 to 4	1.0 cc.	0.5 cc.		0.5 cc.	0.5 cc.		Incomplete hemolysis; unit.
5	1 to 6	1.0 cc.	0.5 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.
6	1 to 8	1.0 cc.	0.5 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.
7	1 to 12	1.0 cc.	0.5 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.
8	1 to 16	1.0 cc.	0.5 cc.		0.5 cc.	0.5 cc.		Complete hemolysis.
9	—	1.0 cc.	1.0 cc.		0.5 cc.	0.5 cc.		Complete hemolysis (control)
10	—	—	2.5 cc.		—	0.5 cc.		No hemolysis (control).

Titration of Antigen.—Each antigen should be titrated for anticomplementary activity as shown in Table 69, the hemolytic system,

titration of hemolysin and complement, etc., being exactly as described in Chapter XXXI.

The smallest dose of antigen interfering with hemolysis is the anticomplementary unit and in the titration of antiserum and the complement-fixation tests is used in one-half of this amount. Extracts keep remarkably well in a refrigerator but when used at long and irregular intervals should be retitrated before use.

A titration for hemolytic activity is not required.

Titration of Antiserum.—Each antiserum requires titration with its homologous or corresponding antigen of meat extract.

One-tenth cubic centimeter of serum (0.2 cc. of serum-glycerin) is diluted to 1 cc. with saline solution, heated at 62° C. for thirty minutes and further diluted with 19 cc. of saline to give a 1 to 200 dilution.

The complement is titrated in the presence of one-half of the anticomplementary unit of corresponding meat extract antigen.

The arrangement of the test is shown in Table 70, the hemolytic system being exactly the same as in the syphilis complement-fixation test.

TABLE 70.—TITRATION OF ANTISERUM.*

Tube.	Antiserum 0.5 cc.	Meat extract.†	Complement 2 full units.		Hemolysin 2 units.	Corpuscles 2 per cent.		Readings.
1	1 to 200	0.5 cc.	1.0 cc.	Refrigerator at 6 to 8° C. for 15 to 18 hours followed by water-bath for 10 to 15 minutes.	0.5 cc.	0.5 cc.	Water-bath one hour.	++++
2	1 to 300	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		++++
3	1 to 400	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		++++ (*unit*)‡
4	1 to 600	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		+++
5	1 to 800	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		++
6	1 to 1200	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		++
7	1 to 1600	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		+
8	1 to 2400	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		−
9	1 to 3200	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		−
10	1 to 4800	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		−
11	0.5 cc. saline	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.		− (antigen control)
12	1.0 cc. saline	—	1.0 cc.		0.5 cc.	0.5 cc.		− (hemolytic control)

* Extract of uncooked meat tested with antiserum for uncooked meat. A duplicate test is required with an extract of cooked meat and its antiserum.

† Dose one-half of the anticomplementary unit so diluted as to be contained in 0.5 cc.

‡ Highest dilution or smallest amount of serum giving a ++++ reaction. In the main tests 4 units or 0.5 cc. of 1 to 100 dilution would be used.

The smallest amount of antiserum giving a ++++ reaction is the unit. As stated above this should be at least 0.5 cc. of 1 to 200 dilution to be satisfactory since a dose of 2 to 10 units is used in the complement-fixation test and should not exceed 0.5 cc. of 1 to 100 dilution in order to avoid the non-specific reactions sometimes yielded by normal rabbit sera. The following examples show the method:

Unit 0.5 cc. of 1 to 200 . . . . . dose 0.5 cc. of 100 (2 units)
Unit 0.5 cc. of 1 to 300 . . . . . dose 0.5 cc. of 100 (3 units)
Unit 0.5 cc. of 1 to 400 . . . . . dose 0.5 cc. of 1 to 100 (4 units)
Unit 0.5 cc. of 1 to 600 . . . . . dose 0.5 cc. of 1 to 100 (6 units)
Unit 0.5 cc. of 1 to 800 . . . . . dose 0.5 cc. of 1 to 100 (8 units)
Unit 0.5 cc. of 1 to 1200 . . . . . dose 0.5 cc. of 1 to 120 (10 units)
Unit 0.5 cc. of 1 to 1600 . . . . . dose 0.5 cc. of 1 to 160 (10 units) etc.

As a general rule the unit remains quite constant over long periods but if the tests are done at irregular intervals, retitrations are indicated.

The Complement-fixation Test.—This is set up as shown in Table 71 in which an extract of sausage was tested for dog, cat and hog meat and found to contain dog and hog. Probably positive reactions would likewise have occurred with a beef extract since this meat is almost invariably put into sausages.

1. Each antigen is used in three doses corresponding to one-half, one-fourth and one-eighth of the anticomplementary unit in order to guard against possible error due to prezone reactions.

2. The hemolysin is titrated as shown on page 392 and used in a dose of 2 units in the complement-titrations and the main test.

3. The complement is titrated in the presence of *each antigen* in a dose of one-half of the anticomplementary unit and used in a dose of 2 full units, so diluted as to be contained in 1 cc. It is not necessary to titrate in the presence of the one-quarter and one-eighth doses of antigen.

TABLE 71.—THE COMPLEMENT-FIXATION TEST FOR THE DETECTION OF MEAT ADULTERATION IN SAUSAGE.

Tube	Extracts 0.5 cc.	Heated antiserum 0.5 cc.*	Complement (2 full units).		Hemolysin 2 units.	Corpuscles 2 per cent.	Water-bath one hour. results.
1	½ unit of sausage	Antidog	1.0 cc.	Refrigerator 6 to 10° C. for 15 to 18 hours followed by water-bath 10 to 15 minutes.	0.5 cc.	0.5 cc.	++
2	¼ unit of sausage	Antidog	1.0 cc.		0.5 cc.	0.5 cc.	++++
3	⅛ unit of sausage	Antidog	1.0 cc.		0.5 cc.	0.5 cc.	++++
4	½ unit of sausage	Anticat	1.0 cc.		0.5 cc.	0.5 cc.	−
5	¼ unit of sausage	Anticat	1.0 cc.		0.5 cc.	0.5 cc.	−
6	⅛ unit of sausage	Anticat	1.0 cc.		0.5 cc.	0.5 cc.	−
7	½ unit of sausage	Antihorse	1.0 cc.		0.5 cc.	0.5 cc.	−
8	¼ unit of sausage	Antihorse	1.0 cc.		0.5 cc.	0.5 cc.	−
9	⅛ unit of sausage	Antihorse	1.0 cc.		0.5 cc.	0.5 cc.	−
10	½ unit of sausage	Antihog	1.0 cc.		0.5 cc.	0.5 cc.	++++
11	¼ unit of sausage	Antihog	1.0 cc.		0.5 cc.	0.5 cc.	++++
12	⅛ unit of sausage	Antihog	1.0 cc.		0.5 cc.	0.5 cc.	++++
13	½ unit of dog	Antidog	1.0 cc.		0.5 cc.	0.5 cc.	++++ (control)
14	½ unit of cat	Anticat	1.0 cc.		0.5 cc.	0.5 cc.	++++ (control)
15	½ unit of hog	Antihog	1.0 cc.		0.5 cc.	0.5 cc.	++++ (control)
16	½ unit of sausage	0.5 cc. saline	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
17	½ unit of dog	0.5 cc. saline	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
18	½ unit of hog	0.5 cc. saline	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
19	0.5 cc. saline	Antidog	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
20	0.5 cc. saline	Anticat	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
21	0.5 cc. saline	Antihog	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
22	1.0 cc. saline	..	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
23	2.5 cc. saline	..	..		..	0.5 cc.	No hemolysis (control)

* Carrying 2 to 10 units.

4. As many antisera are used as one wishes to test for different meats.

5. Duplicate tests are sometimes required in which the antisera for *cooked* meats are used since, for example, an extract of a sausage containing cooked dog flesh may not give complement-fixation with an antiserum prepared by immunizing rabbits with uncooked dog flesh and serum.

6. Known meat extract controls are included for each antiserum as shown in Tubes 13, 14 and 15.

7. Each extract is controlled for anticomplementary activity in the maximum dose, as shown in Tubes 16, 17 and 18, and should show complete hemolysis.

8. Each antiserum is controlled for anticomplementary activity as shown in Tubes 19, 20 and 21 and should show complete hemolysis.

9. A hemolytic system (Tube 22) and a corpuscle control (Tube 23) are included.

THE COMPLEMENT-FIXATION TEST FOR THE DETECTION OF MILK ADULTERATION.

Preparation of Antigen.—This is ordinarily prepared by removing the fat, heating at 60° C. for one hour and adding 5 cc. of 5 per cent phenol to each 95 cc. as a preservative (0.25 per cent). I have tried solutions of the purified caseins but the results were not superior to antigens of whole fat-free milk just described.

Preparation of Antisera.—These are sometimes difficult to prepare and the milk (cow, goat, human, etc.) should be collected with care to keep down the bacterial content as much as possible. After removal of the cream by centrifuging, the milk may be heated at 60° C. for one hour and preserved with phenol as described. This amount of heating does not alter the antigenic specificity of the proteins and enough may be prepared and kept in a refrigerator for the immunization of rabbits. *For the detection of boiled milk, rabbits would require immunization with boiled milk antigens.*

Several animals should be injected with each milk as the mortality may be high before sufficiently good antisera are secured. An excellent method is to give a daily intravenous injection of 0.4 cc. diluted to 1 cc. with saline for 21 to 28 injections. Another good method is to give a weekly intravenous injection of the following amounts: 1, 2, 4, 6, 8 and 10 cc. The mortality from intraperitoneal injections is likely to be lower and the doses may be somewhat higher as 2, 4, 6, 8, 10 and 15 cc.

One week after the last injection blood is removed from the ear of each animal and the serum tested with homologous antigen for its antibody content. The smallest amount giving a strongly positive reaction should not be less than 0.5 cc. of 1 to 200 of serum; otherwise 2 or 3 more injections should be given.

Titration of Antigen.—Each milk antigen should be tested for its anticomplementary activity and the arrangement is similar to that shown in Table 69. Undiluted milk is sometimes hemolytic. The first tube or two may require centrifuging to determine the question of hemolysis in view of the cloudy state of the contents.

Titration of Antiserum.—A portion of each antiserum is heated at 62° C. for one-half hour and titrated with its corresponding antigen in dose of one-half of its anticomplementary unit as shown in Table 70. The smallest ++++ amount should be at least 0.5 cc. of 1 to 200 because at least 2 to 10 units should be used in the complement-fixation test providing the dose does not exceed 0.5 cc. of 1 to 100 of serum in order to avoid the non-specific reactions sometimes yielded by normal rabbit serum. These immune sera may be preserved unheated with an equal part of glycerin and heated just before use; the antibody remains quite constant after the initial drop.

The Complement-fixation Test.—The arrangement is shown in Table 72 in which a mixture of cow and human milks (marked as "unknown") was examined. Well marked reactions occurred with the antigens of cow and human milk but a weak reaction also occurred with goat milk antigen, probably because of a group reaction between antigoat antibody and cow's milk.

TABLE 72.—THE COMPLEMENT-FIXATION TEST FOR THE DETECTION OF MILK ADULTERATION.

Tube.	Milk antigens 0.5 cc.	Heated antiserum 0.5 cc.*	Complement (2 full units).		Hemolysin 2 units.	Corpuscles 2 per cent.	Water-bath one hour results.†
1	½ unit unknown	Anticow	1.0 cc.	Refrigerator 6° to 10° C. for 15 to 18 hours followed by water-bath for 10 to 15 minutes	0.5 cc.	0.5 cc.	+++
2	¼ unit unknown	Anticow	1.0 cc.		0.5 cc.	0.5 cc.	++++
3	⅛ unit unknown	Anticow	1.0 cc.		0.5 cc.	0.5 cc.	+++
4	½ unit unknown	Antigoat	1.0 cc.		0.5 cc.	0.5 cc.	+
5	¼ unit unknown	Antigoat	1.0 cc.		0.5 cc.	0.5 cc.	−
6	⅛ unit unknown	Antigoat	1.0 cc.		0.5 cc.	0.5 cc.	−
7	½ unit unknown	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	++
8	¼ unit unknown	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	+++
9	⅛ unit unknown	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	++
10	½ unit cow	Anticow	1.0 cc.		0.5 cc.	0.5 cc.	+++ (control)
11	½ unit goat	Antigoat	1.0 cc.		0.5 cc.	0.5 cc.	+++ (control)
12	½ unit human	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	+++ (control)
13	½ unit unknown	0.5 cc. saline	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
14	½ unit cow	0.5 cc. saline	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
15	½ unit goat	0.5 cc. saline	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
16	½ unit human	0.5 cc. human	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
17	0.5 cc. saline	Anticow	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
18	0.5 cc. saline	Antigoat	1.0 cc.		0.5 cc.	0.5 cc.	− (contraol)
19	0.5 cc. saline	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
20	1.0 cc. saline	..	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
21	2.5 cc. saline	..	..		..	0.5 cc.	No hemolysis (control)

* Carrying 2 to 10 units.
† Cloudy tubes should be centrifuged if necessary to determine question of hemolysis.

1. Each milk antigen is used in dose of one-half, one-fourth and one-eighth the anticomplementary units, so diluted that each amount is contained in 0.5 cc.

2. Hemolysin is titrated as shown on page 392.

3. Complement is titrated in the presence of one-half the anticomplementary unit of each antigen and used in dose of 2 full units.

4. A portion of each antiserum is heated at 62° C. for one-half hour and so diluted that 0.5 cc. carries from 2 to 10 units providing

that the total amount of serum does not exceed 0.5 cc. of 1 to 100 dilution.

5. The usual positive controls (Tubes 10, 11 and 12) are included as likewise controls on the anticomplementary activity of each antigen (Tubes 13, 14, 15 and 16) and antiserum (Tubes 17, 18 and 19). Hemolytic (Tube 20) and corpuscle (Tube 21) controls are included.

THE COMPLEMENT-FIXATION TEST FOR THE DETECTION OF SEMEN.

This test is sometimes requested in medico-legal cases involving the charge of rape when microscopic examinations of the material for spermatozoa are unsatisfactory. It yields very satisfactory results although stains of vaginal secretions free from spermatozoa, human blood, albuminous urine and sputum may yield confusing cross complement-fixation reactions with anti-spermatozoa sera unless carefully controlled.

Preparation of Extract Antigens.—As a general rule stains on clothing are submitted for the test. These should be carefully cut away with *perfectly clean* scissors and extracts prepared in exactly the same manner as described on page 419 for the preparation of extracts of blood stains. The stock extract should be approximately a 1 to 25 solution and in the case of shirts and underwear pieces aggregating 3 to 4 inches square may be extracted with about 25 cc. of phenolized saline solution.

A control extract is also advisable prepared by extracting *unstained* portions of the same garment in the same manner.

A known semen control antigen should be prepared at the same time by diluting 1 cc. with 24 cc. of phenolized saline.

Preparation of Antiserum.—As a general rule only a known anti-human spermatozoa serum is required prepared by giving rabbits a weekly intravenous injection of the following amounts of human semen: 0.5, 1, 1.5, 2, 2 and 2 or 3 cc.; each dose should be diluted with an equal part of sterile saline solution. A stock solution of semen may be prepared by diluting with an equal part of saline carrying 0.5 per cent of phenol, kept in the refrigerator and used for this immunization of rabbits and as a known positive control antigen in the complement-fixation tests.

Rabbits stand the injections quite well. One week after the last dose, blood is removed from ear veins and the serum titrated. The smallest amount giving a ++++ reaction with an antigen of human semen in one-half of its anticomplementary unit, should not exceed 0.5 cc. of 1 to 200 dilution since 2 to 10 units are used and the dose should not be more than 0.5 cc. of 1 to 100 dilution to avoid

the non-specific reactions sometimes yielded by normal rabbit serum with antigens of semen.

If the antibody content is too low a few more injections should be given. If satisfactory the animals are bled and the unheated sera preserved in a refrigerator with an equal part of chemically pure glycerin. Before the preservative is added the group antibodies for human blood serum and vaginal secretions may be removed by adding to each 10 cc. of antisemen serum, 5 cc. of 1 to 100 dilution of human serum and 5 cc. of a heavy saline vaginal washing from a child with vaginitis; after standing one hour at room temperature the mixture is thoroughly centrifuged and the supernatant serum preserved. Of course the titer is now reduced at least one-half by reason of the dilution.

Titration of Extracts.—The extracts of stained and unstained clothing and of human semen are titrated for anticomplementary activity in exactly the same manner as shown in Table 69 and as described on page 421 for the titration of blood stains.

Titration of Antiserum.—A portion of antisemen serum is heated at 62° C. for one-half hour an titrated for its antibody content in the same manner as shown in Table 70, the antigen being one-half of the anticomplementary unit of human semen. The smallest amount of serum giving a ++++ reaction is the unit and in the complement-fixation tests 2 to 10 units are employed providing the dose does not exceed 0.5 cc. of 1 to 100 dilution of serum in order to avoid non-specific reactions.

The Complement-fixation Test.—This is arranged as shown in Table 73 taken from the protocols of one of my cases showing seminal stains on an undergarment.

TABLE 73.—THE COMPLEMENT-FIXATION TEST FOR THE IDENTIFICATION OF SEMINAL STAINS.

Tube	Extracts 0.5 cc.	Heated antiserum.	Complement (2 full units).		Hemolysin 2 units.	Corpuscles 2 per cent.	Water-bath one hour results.
1	½ unit unknown*	0.5 cc.‡,	1.0 cc.	Refrigerator 6 to 10° C. for 15 to 18 hours followed by water-bath for 10 to 15 minutes.	0.5 cc.	0.5 cc.	+++
2	¼ unit unknown	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	+++
3	⅛ unit unknown	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	+
4	½ unit of control*	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	−
5	¼ unit of control	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	−
6	⅛ unit of control	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	−
7	½ unit semen†	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	++++ (control)
8	¼ unit semen	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	++++ (control)
9	⅛ unit semen	0.5 cc.	1.0 cc.		0.5 cc.	0.5 cc.	++++ (control)
10	½ unit unknown	0.5 cc saline	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
11	½ unit of control	0.5 cc. saline	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
12	½ unit of semen	0.5 cc. saline	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
13	0.5 cc. saline	0.5 cc. antiserum	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
14	1.0 cc. saline	..	1.0 cc.		0.5 cc.	0.5 cc.	− (control)
15	2.5 cc. saline	..	..		..	0.5 cc.	No hemolysis (control)

* Was not anticomplementary in 0.5 cc. of undiluted extract. The doses used therefore were 0.5 cc. of 1 to 2, 1 to 4 and 1 to 8 of stock extract.

† Anticomplementary unit was 0.5 cc. of 1 to 4; was used in dose of 0.5 cc. of 1 to 8, 1 to 16 and 1 to 32.

‡ Carrying 2 to 10 units.

1. The extract of stain is listed in the table as "unknown." It happened to be free of anticomplementary activity and was used therefore in dose of 0.5 cc. of 1 to 2, 1 to 4 and 1 to 8 of the stock solution. The extract of unstained cloth is listed in the table as "control" and was used in the same amounts. The anticomplementary unit of the extract of semen was 0.5 cc. of 1 to 4; $\frac{1}{2}$ unit was therefore, 0.5 cc. of 1 to 8, etc.

2. The unit of antiserum was 0.5 cc. of 1 to 400. Four units or 0.5 cc. of 1 to 100 were used, the serum being previously heated at 62° C. for thirty minutes.

3. The hemolysin was titrated as described on page 392.

4. The complement was titrated in the presence of each extract as described on page 393 and used in dose of 2 full units.

5. Strongly positive reactions were observed with the extract of stains (Tubes 1, 2 and 3); negative reactions occurred with the control extract (Tubes 4, 5 and 6). Strongly positive reactions also occurred with the known semen antigen (Tubes 7, 8 and 9; positive controls). Each extract was tested for anticomplementary activity in Tubes 10, 11 and 12 and gave complete hemolysis, also the antiserum (Tube 13). Tube 14 was the hemolytic system control and gave complete hemolysis; Tube 15 was the corpuscle control which gave no hemolysis. Subsequent information confirmed the results that the stains were of semen about twenty-seven days old.

6. In medico-legal cases it is also advisable to include control extracts of vaginal secretions taken from a child with vaginitis, albuminous urine and saliva in case it is claimed that stains may be of these substances. If blood stains are present positive reactions could occur with antisemen serum unless the group antibody had been removed by absorbing the antiserum with human serum as described above.

CHAPTER XXXVIII.

TECHNIQUE OF METHODS FOR CONDUCTING COMPLEMENT-FIXATION TESTS WITH ANTI-COMPLEMENTARY SERA, SPINAL FLUIDS, EXUDATES AND TRANSUDATES.

As is well known sera, spinal fluids, exudates and transudates (chancre secretions, pleural, pericardial and peritoneal exudates and transudates) may become anticomplementary or capable of themselves of fixing or inactivating complement in the *absence* of antigen; this is especially true of old and contaminated sera, chancre and pleural exudates, by reason of the presence of bacteria. In routine complement-fixation tests the phenomenon is detected by the occurrence of incomplete hemolysis in the serum controls. Since any serum, spinal fluid, etc., may contain anticomplementary substances, one should never assume that they are absent but always and invariably include the serum controls in every complement-fixation test.

The nature and mechanism of the phenomenon have been discussed in Chapter VII and in this chapter I wish to describe methods for testing anticomplementary fluids because it is not always possible or convenient to retest with fresh fluids.

First Method.—In the first place whenever a large number of sera show incomplete hemolysis of the controls in routine work it is entirely unlikely that the sera are anticomplementary. In my experience the trouble is usually with the complement. It may be that the unit of hemolysin is the same as usual and thereby indicating that the complement possesses the usual degree of hemolytic activity; the complement unit may be likewise the usual 0.25 to 0.3 cc. of 1 to 30 dilution but is apt to be a little higher like 0.35 to 0.4 cc. But yet in the tests employing 2 full units some of the controls may show incomplete hemolysis indicating that the complement is supersensitive to the slight anticomplementary activity which almost all sera possess. When tests are conducted routinely three times a week trouble of this kind may be expected once or twice a year and especially during the summer months. It probably has some relation to diet and when ever dealers have sold me guinea-pigs previously used, as for antitoxin titrations or tuberculosis tests, the complement sera have been particularly likely to behave in this manner regardless of the fact that the animals appeared to be in good health.

In the second place it is possible to safely read some anticomplementary reactions *when the serum controls show but slight interference with hemolysis.* The following are examples in which *negative* reports were correctly rendered:

1 – – – – 1
2 – – – – 1
2 1 – – – 2
2 – – – – 2
4 – – – – 2
4 – – – – 3

In other words if the first tube shows the same or very nearly the same degree of inhibition of hemolysis as the serum control with none or but the slightest inhibition in the second tube it is practically certain that the serum contains no antibody and that a negative report may be rendered; indeed, the evidence of a negative reaction is even stronger under these conditions than if the serum were free of anticomplementary action because if even a trace of antibody were present, strong inhibition of hemolysis would occur in the second and even the third tubes of the quantitative test.

Positive reports are also possible of correct reading and especially by *experienced* serologists *whenever the serum control shows less than* +++ (3) reactions, although it is impossible to estimate the degree of positiveness (one reporting only a "positive" reaction):

4 4 3 – – 2
3 2 – – – 1
4 1 – – – 1
4 4 4 2 – 2

It is true, however, that some risk of error may accompany reports of this kind and it is always advisable to repeat the tests; this is especially true in the case of inexperienced workers.

Whenever the serum control shows complete inhibition of hemolysis (++++ or 4) I believe it is too risky to report, as, for example, in the following reactions:

4 4 1 – – 4
4 2 – – – 4
4 4 3 – – 4

It is usually true that a 4 4 4 3 – 4 reaction is positive but whenever a serum is so anticomplementary that it gives a 4 reaction in the control it carries with it the possibility of giving strong inhibitions of hemolysis in the 0.05 and 0.025 cc. doses (second and third tubes of the quantitative test) and even in the 0.005 cc. dose or fourth tube since *the presence of antigen frequently intensifies the anticomplementary effects of serum* in the absence of antibody.

Second Method.—Sera *deeply* stained with hemoglobin are always likely to be anticomplementary; likewise serum, spinal fluid, urine, milk, etc., containing large numbers of bacteria, and freshly collected chancre exudates.

When these are tested it is advisable to put up a control on each

dose; this is readily accomplished in routine work by setting up two series of tubes in the usual manner; antigen is added to all tubes of the first row except No. 6 (serum control) but not to the second row which serves as the controls. The sixth tube of the second row could be omitted as it is a duplicate of No. 6 in the first row but I usually include it in order not to disturb the usual routine. The following are examples of reactions employing the quantitative test:

First row:	4 4 4 1 – 4	Syphilitic serum
Second row:	4 1 – – – 4	
First row:	4 4 4 1 – 3	Syphilitic serum
Second row:	4 1 – – – 4	
First row:	4 4 1 – – 2	Syphilitic spinal fluid
Second row:	3 – – – – 3	
First row:	4 2 – – – 4	Non-syphilitic serum
Second row:	4 1 – – – 4	
First row:	3 2 – – – 2	Non-syphilitic serum
Second row:	3 1 – – – 3	
First row:	4 4 2 – – 4	Non-syphilitic spinal fluid
Second row:	4 3 1 – – 4	

Whenever complement fixation in the first row is *markedly* greater than in the second row as shown above with the two syphilitic sera and spinal fluid one may safely report a positive reaction without attempting to express an opinion on the degree of positiveness; negative reactions may be reported when the differences between the first and second rows are very slight. But whenever the differences are less marked it is unsafe and unwise to vouchsafe a positive report as, for example, in reactions of these kinds conducted with known sera allowed to become anticomplementary:

First row:	4 4 4 2 – 4	Syphilitic serum
Second row:	4 4 1 – – 4	
First row:	3 1 – – – 2	Syphilitic serum
Second row:	2 – – – – 2	
First row:	2 1 – – – 1	Syphilitic serum
Second row:	1 – – – – 1	
First row:	4 4 4 2 1 4	Syphilitic serum
Second row:	4 4 4 1 – 4	
First row:	4 3 2 – – 4	Syphilitic spinal fluid
Second row:	4 2 – – – 4	

It is true that the first row carrying antigen almost always shows stronger reactions than the second row but unless the differences are quite marked it is not safe to give a positive report because normal or negative but anticomplementary serum and spinal fluid always gives a greater degree of inhibition of hemolysis in the presence of antigen.

Third Method.—A third method which I sometimes employ is a slight modification of the second method in which each serum is set up in two rows carrying 0.1, 0.05, 0.025, 0.0125, 0.006 and 0.003 cc. of serum.

These are arranged by placing 0.8 cc. of saline solution in the first tube of each row and 0.5 cc. in the remaining five tubes. To the first tube of each row is added 0.2 cc. of serum, mixed and 0.5 cc.

transferred to the second tube; No. 2 is mixed and 0.5 cc. carried to No. 3, etc., to the sixth tube from which 0.5 cc. is discarded. Antigen is added to the tubes of the first row; the second row serves as a control on each dose of serum. The test is otherwise completed in the usual manner.

The following are examples in which *positive* reports were returned:

First row:	4 4 4 2 – –	Syphilitic serum
Second row:	4 1 – – – –	
First row:	4 4 1 – – –	Syphilitic serum
Second row:	3 – – – – –	
First row:	3 2 1 – – –	Syphilitic serum
Second row:	1 – – – – –	

In other words if the degree of fixation in the first row is *markedly* greater than in the second row it is ordinarily safe to render a positive report without expressing any opinion on the degree of positiveness. But if the differences are not marked it is unsafe to report positive reactions; for example the following were observed with known syphilitic sera but reported simply as "anticomplementary:"

First row:	4 4 4 4 3 –	Syphilitic serum
Second row:	4 4 4 2 – –	
First row:	4 4 1 – – –	Syphilitic serum
Second row:	4 3 – – – –	
First row:	3 – – – – –	Syphilitic serum
Second row:	1 – – – – –	

Negative reactions can be reported only when the degree of fixation of complement is slight and about the same in both rows, as per the following examples with known sera:

First row:	4 1 – – – –	Non-syphilitic serum
Second row:	4 – – – – –	
First row:	2 – – – – –	Non-syphilitic serum
Second row:	2 – – – – –	

If the reactions in both rows are strongly positive and practically the same it is unsafe to report negatively because a serum may contain antibody under these conditions. For example the following reactions were observed with syphilitic sera yielding 4 4 3 – – – reactions in the fresh state and allowed to become anticomplementary:

First row:	4 4 4 3 – –	No. 1
Second row:	4 4 3 1 – –	
First row:	4 4 4 1 – –	No. 2
Second row:	4 4 3 – – –	
First row:	4 4 4 4 4 1	No. 3
Second row:	4 4 4 2 1 –	

Sachs Method.—As stated in Chapter VII Sachs has described a very useful method for testing anticomplementary sera. I have used it during the past two years with considerable success and satisfaction and especially with sera contaminated with bacteria. Sera very deeply tinged with hemoglobin do not respond quite as well to the method. Rabbit, dog and the sera of the lower animals may

be treated in the same manner since it likewise removes the anti-complementary substances from the majority but not the substance responsible for the non-specific complement-fixation reactions sometimes yielded by the normal sera of rabbits, dogs and mules. The method has not been applied to spinal fluids.

The technique as applied to my method is as follows:

1. Heat 0.5 cc. of serum at 55° C. for fifteen minutes.
2. Add 4.1 cc. of accurately titrated $\frac{N}{300}$ hydrochloric acid and mix.
3. After standing one-half hour at room temperature centrifuge thoroughly and discard the sediment.
4. To the supernatant fluid add 0.4 cc. of 10 per cent sodium chloride solution. The acid is fixed by the precipitate of globulin; hence neutralization is unnecessary.
5. This gives a 1 to 10 dilution of original serum ready for testing.
6. For the *quantitative test* arrange the usual six tubes and place saline solution as follows:

No. 1 : none	No. 4 : 2.0 cc.
No. 2 : none	No. 5 : 0.5 cc.
No. 3 : 0.5 cc.	No. 6 : none.

7. In Tubes Nos. 1 and 6 place 1 cc. of prepared 1 to 10 serum; place 0.5 cc. in No. 2; place 0.5 cc. in No. 3, mix and transfer 0.5 cc. to No. 4; mix, transfer 0.5 cc. to No. 5 and discard 1.5 cc.; mix No. 5 and discard 0.5 cc. The doses are 0.1, 0.05, 0.025, 0.005 and 0.0025 cc. with 0.1 cc. in the serum control as in the usual test except that the total volume in Tubes Nos. 1 and 6 are 0.5 cc. more at the end of the test than in the usual method.
8. For the *qualitative* test it is only necessary to place 1, 0.5 and 1 cc. respectively in three tubes, the third being the serum control.
9. The test is now completed in the usual manner.

The following are examples showing successful and unsuccessful results taken from a large number of comparative tests before and after treatment by Sachs method and conducted for me by Miss Yagle in the course of routine work:

Source.	Before.	Sachs method.
Syphilitic	4 4 4 – – 4	4 – – – – 1
Syphilitic	4 4 3 – – 3	4 4 – – – –
Syphilitic	4 4 4 4 4 4	4 4 4 – – 2
Syphilitic	4 4 4 4 4 3	4 4 4 3 – –
Syphilitic	4 3 1 – – 3	2 1 – – – –
Syphilitic	4 4 3 – – 4	4 3 – – – 1
Syphilitic	4 4 4 1 – 4	4 4 4 – – –
Syphilitic	4 3 1 – – 4	4 2 – – – –
Normal	3 2 – – – 2	– – – – – –
Normal	3 – – – – 2	– – – – – –
Normal	2 1 – – – 2	– – – – – –
Normal	4 4 4 4 3 4	4 4 1 – – 4
Normal	4 4 3 – – 4	4 4 1 – – 4
Normal	4 3 1 – – 4	– – – – – –
Normal	4 4 4 4 4 3	2 – – – – 2
Normal	3 1 – – – 2	– – – – – –

It will be noted that the method does not remove the anticomplementary substances from all sera and especially those containing unusually large amounts but in the majority of sera enough is removed to make it possible to safely test the sera insofar as positive or negative reactions are concerned.

Other Methods have been likewise proposed from time to time for testing anticomplementary sera and spinal fluids. One of the best of these is a modification of the McIntosh-Fieldes method in which the serum is tested in a fixed dose of 0.1 cc. with increasing amounts of complement like, 2, 3, 4, 6, 8 units, etc.; a similar series of serum controls without antigen are included. In this manner it is possible to measure the amount of complement absorbed by serum and antigen and by serum alone and when the differences are marked a positive report may be rendered; when these amounts are the same or but slightly different, a negative report is usually possible. I have used the method with much success but have not adopted it for routine work because of the large amounts of complement serum ordinarily required.

Dr. W. E. Gibb of Seattle has stated that he has successfully used a method with strongly positive anticomplementary sera consisting of preparing the usual dilutions of my quantitative method, adding 0.5 cc. of 1 to 30 complement to each, incubating at 37° C. for one-half hour, heating at 56° C. for fifteen minutes and finishing up in the usual manner. This technique is based upon the assumption that the anticomplementary state is due to the presence of a natural "antigen" in the serum which is exhausted in the method.

TECHNIQUE OF COMPLEMENT-FIXATION TESTS WITH CHANCRE EXUDATES.

Since complement-fixation tests with chancre exudates are sometimes successful in the diagnosis of syphilis during the primary stage before the blood serum yields positive reactions as originally described by Klauder and myself, it may be well to describe the technique at this place since these fluids are almost always slightly anticomplementary. My method is as follows:

1. The fluid is heated in the usual manner at 55° C. for fifteen minutes. If the quantity is less than 0.8 cc. sterile saline solution is now added to this amount.

2. In a series of two tubes are placed 0.3 and 0.1 cc.; similar amounts are placed in a rear or second series of tubes. If a larger amount of fluid is available the doses may be larger in both series as 0.5 and 0.2 cc.

3. Saline is added to bring the total volume to 0.5 cc. in all tubes of the first row and to 1 cc. in the second row.

4. The usual 10 units of antigen in 0.5 cc. are added to the tubes of the first row; none is added to the second row, which are the serum controls on each dose.

5. The usual 2 full units of complement are now added to all tubes followed by the routine incubation of sixteen to eighteen hours at 6° to 10° C. and ten to fifteen minutes in a water-bath, etc., as described in Chapter XXXI.

The following are examples of reactions:

First row:	4 2	Positive
Second row:	1 –	
First row:	2 1	Positive
Second row:	– –	
First row:	3 1	Positive
Second row:	1 –	
First row:	1 –	Doubtfully positive
Second row:	– –	
First row:	2 –	Negative
Second row:	2 –	
First row:	1 –	Negative
Second row:	1 –	

TECHNIQUE OF COMPLEMENT-FIXATION REACTIONS WITH URINE, MILK, TRANSUDATES AND EXUDATES.

Transudates like pleural, pericardial, peritoneal and joint fluids are usually free of anticomplementary activity and may be tested in the same manner as serum described in Chapter XXXI. They should be heated at 55° C. for fifteen minutes. As a general rule, however, their antibody content is less than in serum and larger doses are sometimes required similar to those employed in testing spinal fluid (0.5, 0.25, 0.125 cc. etc.)

Exudates like blister fluids and tuberculous pleural exudates are much more likely to be anticomplementary; likewise urine and milk.

Blister fluids may be tested as described above for chancre exudates since the amounts submitted are usually quite small.

Urine, *milk* and *exudates* (like pleural exudates for tuberculosis) should be freshly collected and kept at a low temperature until examined. Each should be heated at 55° C. for fifteen minutes and first tested for anticomplementary activity as follows:

1. Titrate hemolysin.
2. In a series of 8 test-tubes place 0.5 cc. undiluted, 1 to 2, 1 to 3, 1 to 4, 1 to 6, 1 to 8, 1 to 12 and 1 to 16 dilutions of the fluid to be tested. Titrate the complement and add full 2 units (1 cc.); also 0.5 cc. of saline solution.
3. Water-bath at 37° C. for one hour.
4. Add 2 units of hemolysin and 0.5 cc. of 2 per cent corpuscles.
5. Water-bath one hour and read.
6. Include a hemolytic system and corpuscle control.

The smallest amount giving even slight interference of hemolysis is the anticomplementary unit and varies greatly with different specimens.

7. In setting up the complement-fixation tests place one-fourth of the anticomplementary unit in a front and rear tube; for example if this happens to be 0.5 cc. of 1 to 4 dilution use 0.5 cc. of 1 to 16.

8. To the front tube add the usual dose of antigen and to the rear tube 0.5 cc. of saline (control).

9. The complement is titrated in the presence of the antigen in the usual manner and 2 full units added to both tubes.

10. The primary incubation is the usual fifteen to eighteen hours at 6° to 10° C. followed by ten to fifteen minutes in a water-bath, the test being finished in the usual manner as described in Chapter XXXI.

PART IV.

CLINICAL APPLICATIONS OF COMPLEMENT-FIXATION TESTS.

CHAPTER XXXIX.

METHODS FOR OBTAINING BLOOD, CHANCRE EXUDATE AND SPINAL FLUID FOR COMPLEMENT-FIXATION TESTS.

Complement-fixing antibodies are ordinarily widely distributed throughout the body fluids and the syphilis complement-fixing substance may be detected not only in the plasma and serum of the blood but likewise in the cerebrospinal fluid, milk, urine, various transudates like synovial, pericardial, peritoneal and pleuritic effusions as well as in inflammatory exudates and especially chancre secretions and blister fluids. Blood serum and cerebrospinal fluid, however, are mostly employed and there is no particular advantage in employing plasma.

INFLUENCE OF METHODS FOR THE COLLECTION OF BLOOD.

Source of Blood.—Blood for the syphilis and other complement-fixation tests is generally collected by venipuncture, but with the introduction of modifications of the Wassermann test requiring smaller amounts of serum, the practice of obtaining blood from the finger is sometimes employed.

Comparative complement-fixation tests conducted with sera secured from a number of syphilitic individuals from a finger and a vein at the same time and tested within three days, have shown no differences either in a qualitative or quantitative test; the amount of antibody in venous blood appears to be the same as in a mixture of venous and arterial blood from a finger. If, however, the specimens of blood are kept for more than three days differences may be apparent, that is, sera collected from a finger are more apt to become contaminated with bacteria and develop anticomplementary properties.

The antibody content of finger and vein blood appears therefore, to be the same and finger blood is just as suitable for the comple-

ment-fixation test as vein blood, if examined before opportunity is afforded for the acquisition of thermostabile antilysins. If many days must elapse from the time of collection of blood to testing of serum, venous blood is preferable *if collected aseptically*; contaminated venous blood is no better than finger blood, as both may develop antilysins in equal degree.

Collection of Blood for the Wassermann Test.—It is claimed by some serologists that the blood serum of non-syphilitic individuals collected during jaundice, certain stages of diabetes mellitus, during the febrile period of malaria, pneumonia and other diseases, may yield falsely positive reactions; in my experience these reactions are very rare and largely preventable by technical details and especially against anticomplementary effect of serum.

Craig and Nichols[1] have stated that the serum of a syphilitic individual collected during or immediately after an acute alcoholic debauch may give a falsely negative reaction; several serologists agree with them and I believe it good practice to avoid this possible source of error.

It is commonly and widely believed that the serum of a syphilitic collected during or immediately after intensive specific treatment may yield a falsely negative reaction; I question the accuracy of this statement. In my experience the results are frequently just the reverse, that is, a serum reacts positively when tested during or immediately after treatment and negatively after a period of some weeks or months thereafter during which specific treatment has not been given. At any rate, one negative reaction is of little value and the test should always be repeated at intervals over a sufficiently long period to make sure of the correctness of a negative reaction.

Blood collected within an hour after a meal is apt to yield a chylous or milky appearing serum; this does not appear to cause harm insofar as complement-fixation reactions are concerned, although some serologists believe that these sera are particularly apt to prove anticomplementary.

While these matters are worthy of attention and further study in relation to the biology and mechanism of the Wassermann reaction, they do not directly concern the technique of collection of blood for the test; in reference to the latter the following technical details are of value in the interests of the best work:

1. If blood is to be sent a distance or, if for other reasons the serum cannot be tested for four or more days, it should be collected aseptically from a vein in order to guard against bacterial contamination, which results in the development of thermostabile anticomplementary substances (antilysins).

2. If the serum is to be tested within three or four days of collection of blood and can be kept at a temperature of 6° C. or colder, the specimen may be collected from finger, skin or vein without

special precautions against contamination, inasmuch as the interval is not generally sufficient for the growth of bacteria to render the serum anticomplementary.

3. If the specimen of blood is subject to agitation as in the mails, the container should be of such size as to be filled, or almost so, with blood, in order to reduce the amount of agitation and consequent hemolysis, inasmuch as an excess of hemoglobin in the serum tends to render it anticomplementary.

Spinal fluids, while collected with aseptic precautions, are not infrequently contaminated with a skin coccus; fortunately they acquire anticomplementary effects rather tardily. *With both blood and spinal fluid regardless of the technique of collection, it is important to keep the specimens at or as near 0° to 2° C. as possible; this is the most important single safeguard for preservation.*

From the laboratory standpoint, I believe *the serum should be left on the clot until ready for testing unless the interval is so long and the temperature such that hemolysis takes place;* in my experience separated sera are more apt to become anticomplementary than sera on the clots, both being kept and handled under identical conditions.

AGE AND ANTICOMPLEMENTARY ACTIVITY OF SERUM AND SPINAL FLUID.

In the course of serological work, sera are sometimes secured by centrifuging defibrinated blood immediately after collection; a point of interest is whether the antibody content is the same as that in serum left on the clot for several to twenty-four or forty-eight hours, inasmuch as the complement content seems to be slightly increased by the latter procedure.

In an extensive study of this subject Watanabe[2] has shown that the antibody concerned in the Wassermann reaction exists in the plasma of the blood, and my results with serum secured immediately after bleeding have been quite similar.

Von Wedel[3] has made the interesting observation that sera from tuberculous individuals may yield negative or weakly positive tuberculosis complement-fixation reactions when tested on the first day after the collection of blood, whereas the same sera give strongly positive reactions seven days later and may continue to give these strongly positive reactions week after week with unvarying regularity. Sera from non-tuberculous persons gave negative reactions under similar circumstances and an explanation has not been offered of the results with tuberculous sera.

I have conducted similar experiments with syphilitic and non-syphilitic sera but with negative results.[4] Not infrequently portions of serum kept at 6° to 8° C. in a refrigerator for ten or more days yielded somewhat stronger reactions, but these were clearly ascrib-

able to the presence of thermostabile anticomplementary substances causing non-specific complement-fixation reactions, or, to the deterioration of a portion of natural antisheep hemolysin.

The effects exerted by the antilysins responsible for the anticomplementary action of sera are very much influenced by natural hemolysins and the results are modified by the balance of power between these two substances in any given serum. With cerebrospinal fluid, the influence of antilysins is not generally so modified because of the absence of natural hemolysins in the majority of fluids, except antisheep hemolysin in the fluids of paretic individuals; or, the hemolysins may be present in but traces and insufficient for influencing the results. Likewise, in the antihuman and antichicken hemolytic systems the influence of antilysins in heated sera are not subject to the influence of natural hemolysins to the same degree as in the antisheep and antiox systems.

These antilysins have therefore, an important influence upon complement-fixation reactions and *when specimens of blood and spinal fluid are sent to a laboratory from a distance, permitting the development of antilysins, the results of complement-fixation tests must vary in some degree from those conducted with perfectly fresh sera and fluids;* this fact should be kept in mind in judging the merits of a technique and explains in part the differences in degree of positive reactions observed with portions of the same blood of a syphilitic person sent to different laboratories in the same or different cities where methods vary in the care with which the specimens are preserved and guarded against the development of antilysins.

Of course, the results should be identical insofar as positive or negative are concerned; only the *degree of positiveness* should be left open to the influence of antilysins. Any serum or spinal fluid so anticomplementary as to jeopardize the correctness of the positive or negative reaction should be detected in the serum control tube and ruled out as unfit.

PRECAUTIONS IN OBTAINING BLOOD.

1. Collection from a vein is advisable and the left arm is preferred.
2. Blood should not be taken immediately after a meal but anytime after one hour is satisfactory. *It is not necessary for the patient to be fasting.*
3. As far as possible avoid drawing blood during febrile periods and acute alcoholism.
4. Sterile containers are advisable although if the test is to be done within three days the presence of a few organisms is without effect.
5. Five cc. of blood is sufficient. *When mailed a small container should be used in order that the contents shall reach almost to the stopper to avoid undue agitation and hemolysis.*

6. *Specimens for mailing should be shipped at once* in order to avoid undue hemolysis and anticomplementary sera. Under these conditions specimens can be shipped long distances.

7. If cotton stoppers are used due care must be taken to prevent blood from coming in contact with them.

8. *Each specimen should be labelled at once* to prevent the very regrettable and inexcusable mistakes sometimes made. Labels may come off and should be held on with rubber bands.

METHODS FOR OBTAINING BLOOD FROM ADULTS AND CHILDREN.

With the Keidel Tube.—One of the most satisfactory methods of obtaining blood from a vein quickly, aseptically and with the least pain is by means of the Keidel tube (Fig. 57). As made at present

FIG. 57.—Securing blood with the Comer-Keidel tube; method of passing the needle. (From Kolmer's "Chemotherapy," W. B. Saunders Co., Publishers.)

by Mr. Comer for the Steel Glass Company of Philadelphia, this very useful apparatus is available with a glass window permitting one to see a flow of blood, and thereby indicating that the vein has been entered, before the neck of the ampoule is broken. The apparatus is dispensed sterilized and ready for use as follows:

1. The patient may sit alongside of a table or desk with the arm extended and elbow supported.

2. The tourniquet is applied above the elbow and the patient requested to open and close the hand vigorously.

3. The skin is wiped with cotton and alcohol.

4. The wire is removed and the hub of the needle grasped (avoiding contamination of the shaft) between the thumb and first finger (Fig. 57) and entered through the skin into the vein at an acute angle to the arm. It is a mistake to enter the needle at an obtuse or right angle because it may be passed through the vein.

5. As the vein is entered there is a peculiar "give-away" sensation readily appreciated by experience; if the "window" type of apparatus is employed a flow of blood is seen.

FIG. 58.—Second step in securing blood with the Comer-Keidel tube. Blood is seen in the "window" indicating that the needle is in the lumen of the vein; the neck of the ampoule is being broken to release the vacuum followed by suction of blood. (From Kolmer's "Chemotherapy," W. B. Saunders Co., Publishers.)

6. The long stem of the ampoule in the rubber is now crushed with the fingers or by a hemostat (Fig. 58) and the ampoule filled with blood by suction owing to the release of the vacuum.

7. If blood does not flow the ampoule may be rotated, in case the lumen of the needle is in contact with the inner wall of the vein. If it is apparent that the needle is not in proper position it may be partly withdrawn and another attempt made, due care being taken not to withdraw the eye of the needle from beneath the skin, which permits the entrance of air, in case the neck of the ampoule has been broken.

8. The tourniquet should be released before the needle is withdrawn to prevent subcutaneous hemorrhage.

9. The site of puncture is compressed for a few seconds and cleansed with alcohol; a dressing is not required.

10. If the blood is to be mailed it is necessary to bend down the rubber tubing and hold it in this position by a rubber band or by tying with a piece of string to prevent leakage of blood or serum in transit.

With a Syringe.—Blood may be also obtained from a vein in the same manner by means of a sterilized 5 or 10 cc. Luer or Record syringe fitted with a No. 20 to 22 needle; it is unnecessary to use a larger needle which inflicts more pain. The needle should be short, not over $1\frac{1}{4}$ inches; long needles interfere with the flow of blood and are more difficult to enter into a vein. The bevel must be short rather than long but not too stumpy; it is almost imperative to use a sharp and rust-free needle to conduct the operation with the minimum of pain and without leaving a stained puncture site in the skin.

After securing the blood the tourniquet should be released before the needle is withdrawn; the blood should be emptied into a sterile test-tube, or better, into a small sterile vial tightly stoppered if the specimen is to be mailed. For this purpose the vial should not be of more than 10 cc. capacity in order to reduce to a minimum the agitation and consequent hemolysis of corpuscles in transit.

The test-tube or vial need not necessarily be sterile if the blood is to be tested within forty-eight hours but if there is a longer interval, the container should be sterilized beforehand by dry heat or it may be boiled for a few minutes, drained and cooled just before use. It is imperative, however, under all circumstances, that the container shall be *chemically clean* and especially free of acids and alkalies which are hemolytic and anticomplementary.

With a Needle.—Blood may be secured from a vein by merely entering a sufficiently large sterilized needle and allowing it to flow into a prepared test-tube or vial. For this purpose the needle should be gauge 18 to 20 since suction is not applied, and the shaft should not be too long as otherwise coagulation may occur. It is of advantage to grasp the needle with a hemostat as shown in Fig. 59. This method, however, is apt to be "messy" and is inferior to the Keidel tube and syringe methods.

By Puncture of a Finger.—From 3 to 5 cc. of blood is readily obtained from a finger and is perfectly acceptable for the Wassermann test. In obese individuals with difficult veins or in nervous individuals who are almost sure to faint if venous puncture is attempted, this method is very valuable although it requires a little more time and patience. The technique is as follows:

1. The hand should be warm. If cold and clammy have the patient immerse the hand in hot water for a few minutes followed by brisk drying.

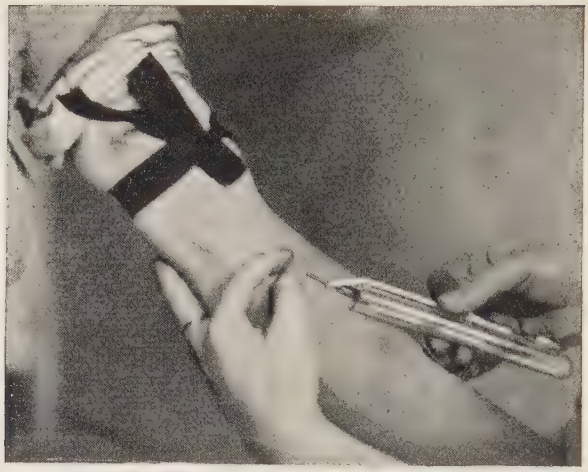

FIG. 59.—Method of withdrawing blood with a needle. (Keen's surgery.)

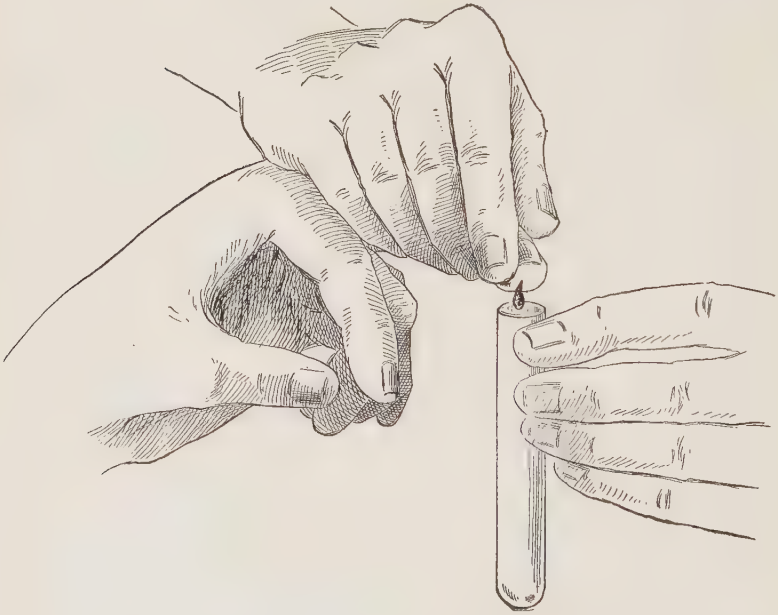

FIG. 60.—Method of securing a small amount of human blood from a finger. By pricking the finger deeply *across* the lines of the skin with a *broad* lancet two or more cubic centimeters of blood are easily collected in a small test-tube. Do not use a large tube, as blood may be lost on the sides of the tube. (From Kolmer's "Chemotherapy," W. B. Saunders Co., Publishers.)

2. Select the middle or ring finger; cleanse with alcohol and dry.

3. Cleanse the broad blade of a Daland blood lancet with alcohol and puncture deeply across the lines of the tip of the finger; there is very little pain.

4. Massage the blood into a *small* test-tube or vial (Fig. 60); it is a mistake to use a large receptacle as blood is wasted on its walls.

5. Cleanse the finger and apply compression for a few seconds. A dressing is not ordinarily required unless bleeding continues.

FIG. 61.—Method of obtaining blood from an external jugular vein. The patient is a child six years of age; shows the position of the patient and manner of distending the vein by pressure above the clavicle. A 5 cc. Record syringe fitted with a No. 20 needle is being used for the withdrawal of blood. The distended vein is painted with tincture of iodine to indicate the position. (Keen's surgery.)

METHODS FOR OBTAINING BLOOD FROM INFANTS.

A few cubic centimeters of blood may be readily obtained from infants by puncturing one of the large toes with a Daland lancet and massaging the blood into a small test-tube, as described above for securing blood by puncture of a finger.

In the case of children from one to six years sufficient blood may be obtained by puncture of one or several fingers. In children over four years of age, a vein may be entered and blood drawn with a Keidel tube or syringe as above described.

Blood may be obtained with a syringe from one of the external jugular veins as shown in Fig. 61 or from a temporal vein.

Blood may be secured from infants under one year of age by puncture of the superior longitudinal sinus as follows:

1. The infant is wrapped in a blanket and the head is steadied by an assistant (Fig. 62).

2. The puncture is made on the median line of the posterior angle of the anterior fontanel.

3. The skin is carefully cleansed. The needle, gauge No. 18, with a short bevel, sterilized and attached to a sterile 5 cc. Record or Luer syringe is passed inward at a right angle for a distance of about 4 mm. and suction made; if blood does not flow the needle should be passed about 2 mm. further, which suffices for the majority of children up to fifteen months of age.

4. At least 3 to 5 cc. of blood may be safely withdrawn and discharged into a vial. The puncture site is then cleansed and may be sealed with a touch of collodion.

FIG. 62.—Method of obtaining blood and giving an intravenous injection by way of the superior longitudinal sinus. The child is six months of age; the shape and size of the anterior fontanel have been outlined; a 5 cc. syringe fitted with No. 18 needle is being employed, the needle having been entered for about 6 millimeters in the median line at the posterior angle and perpendicular to the sinus. (From Kolmer's "Chemotherapy," W. B. Saunders Co., Publishers.)

METHODS FOR OBTAINING BLOOD IN CONNECTION WITH INTRAVENOUS INJECTIONS.

It is a very easy matter to obtain blood just before an intravenous injection is to be given. When administering neoarsphenamin or sulpharsphenamin with a Record or Luer syringe one may have the solution ready for injection in one syringe, but enter the needle attached to a second syringe, remove blood, *loosen the tourniquet*, deftly detach this syringe, attach the second syringe containing the solution and inject. As a general rule, coagulation will not occur before it is possible to empty the blood into a vial.

If the infusion is by gravity the same plan may be followed by

entering the vein and collecting blood in a tube before attaching the tubing, releasing the tourniquet, attaching the tubing and infusing the solution. For this method however, it may be necessary to have assistance.

Not infrequently I pass the needle of a Keidel tube and fill the bulb with blood; then leaving the tube supported on the arm and before releasing the tourniquet, the needle attached to the syringe carrying the solution is entered *above*, the tourniquet released and the injection slowly given when both needles are withdrawn simultaneously. This means two punctures but the needles are small, the pain is almost negligible and both procedures are carried through without the loss of a single drop of blood.

METHOD FOR SECURING CHANCRE EXUDATE.

1. At least 0.2 cc. of exudate is required. This is ordinarily readily obtained by gently squeezing the lesion but if not the chancre may be cleaned off, gently scarified and a few drops of saline solution added followed by gentle squeezing; admixture with a little blood does no harm.

2. The fluid should be drawn into a capillary pipette fitted with a rubber nipple (Fig. 63).

3. After collection the contents may be expelled into a small test-tube for transportations to the laboratory or the tip of the pipette sealed in a flame and the open end stoppered with cork.

FIG. 63.—Capillary tube employed for the collection of chancre exudate.

METHOD FOR OBTAINING SPINAL FLUID.

Apparatus Required and Preparation of Patient.—The *needle* should be gauge 19 to 20, free of rust, with a short bevel and very sharp. Even in the hands of an expert a dull needle with a turned point inflicts unnecessary pain and not infrequently failure to secure spinal fluid. Steel needles may be employed, although some prefer platinum needles because softer and less likely to break in case of violent muscular movements by the patient. My own preference, however, is for the former and I believe it is well to discard a needle or have it sharpened after using it several times. A bright, clean, rust-free, perfectly straight, sharp needle is the first requisite for success in conducting spinal puncture and it is a mistake to use crooked, rusty, dull needles. The bevel should be short rather than long, as otherwise a portion of the fluid may be lost by leakage in the extradural tissues.

Greene has recently advised the use of a gauge 23 needle with a rounded point like a cambric needle to be used with a syringe for suction, because he has observed in experiments that this kind of needle produces the smallest tear in the dura and consequently the least chances for post-puncture drainage of spinal fluid. I have never used a needle smaller than 19 to 20, but am confident that this is a question of considerable importance and feel sure that it is a mistake to use the enormously-sized needles and "trocars" so commonly seen in hospitals.

FIG. 63.—Measuring the spinal fluid pressure with a mercury manometer. (From Kolmer's "Chemotherapy," W. B. Saunders Co., Publishers.)

The needle should always be sterilized by boiling for at least five minutes and the lumen must be freely open and free of rust, coagulated blood, etc. After use, the needle should be again sterilized as spirochetes are not infrequently present in the spinal fluids of syphilitics; after drying it is well to vaselin the stylet lightly to prevent rusting.

A manometer for measuring the cerebrospinal fluid pressure is also advisable and the mercury instrument shown in Fig. 63 has been found quite satisfactory.

A sterile 1 cc. syringe fitted with a No. 26 needle is required for the intracutaneous and subcutaneous injection of sterile 1 per cent solutions of novocaine or butyn for local anesthesia. Two sterile test-tubes for the fluid, tincture of iodine, alcohol, flexible collodion and cotton complete the list of required materials.

The physician should carefully prepare the hands to guard against bacterial infection, or sterilized rubber gloves may be worn. The latter also afford protection against pricking of the fingers and, since the spinal fluid may be infectious from the standpoint of the presence of spirochetes, due care is required, although I do not know of any cases of syphilis caused by this means of contamination.

Preparation of the Patient.—In ambulatory patients the most suitable time for puncture is late in the afternoon to permit resting over night. My practice is to conduct the tests whenever possible during Saturday afternoons which permits most patients to rest in bed over Sunday. It is not necessary to confine the patient for a longer period of time unless headache has followed a previous puncture, in which case a more prolonged stay is advisable.

Special preparation regarding the bowels and diet is not required, although it is well to have had the bowels evacuated and the meal preceding the puncture should be light and taken at least two hours previously. The bladder should be emptied and especially if the patient is nervous.

The skin of the back may be washed with water and soap, followed by alcohol and mercuric chloride solution, but one or two coats of tincture of iodine followed by swabbing with alcohol just before the puncture is made are sufficient.

Site of Puncture.—Spinal puncture should always be done routinely in the lumbar region between the third and fourth or between the fourth and fifth vertebræ. The tip of the cord (conus medullaris) ends between the first and second lumbar vertebræ but extends a little further down in children, so that puncture between the third and fifth vertebræ will not pierce the cord.

If the patient's back is well arched, there is ample space for passage of the needle between the spinous processes of these vertebræ; it is said that in men these processes are usually directed straight backward and that the needle, therefore, may be directed straight in, whereas in women of the same age and build the processes turn downward and the needle consequently must be directed slightly upward.

Choice of Median or Lateral Routes for Puncture.—Some physicians, probably the majority, enter the needle in exactly the middle line between the vertebræ, while others prefer to enter it from $\frac{1}{4}$ to as much as $\frac{1}{2}$ inch to the right or left of the median line directing the needle upward and inward; Regan has recently reviewed the literature and opinions expressed upon this point and prefers the median route. I always use the median route, not only because it is the

easier of the two but for the far more important reason, originally pointed out by Quincke himself many years ago, that the needle is less likely to strike the roots of the cauda equina, since these divide into two laterally placed bundles with a space between them of 2 to 5 cm. This is a point of considerable practical importance because injury of these roots produces not only sharp pain, but has in some instances resulted in more serious motor and sensory disturbances.

When the needle is passed in exactly the middle line, the distance to be traversed before the subarachnoid space and spinal fluid are reached depends upon the age of the patient, the degree of muscular development and panniculus adiposus; in general terms, the distances are as follows, but the matter is of little practical importance, because spinal puncture cannot be performed by passing the needle an arbitrary distance:

Frail adults	4 to 6 cm.
Muscular males	6 to 8 cm.
Obese adults	10 to 12 cm.

Position of the Patient.—As previously stated, spinal puncture is easier with the patient sitting than in the prone position; this is especially true of obese individuals. When the pressure is to be taken the prone position on the left side is preferable, because the variations are less and pressures are always expressed under these conditions unless otherwise specified.

An excellent method is to have the patient sitting astride a chair facing its back with a pillow in the pit of the abdomen and the head resting on the folded arms placed on the back of the chair. The position should be perfectly straight without tilting of the pelvis and the back should be arched to give the maximum of space between the vertebral spaces (Fig. 64). The patient may also sit on the edge of a table or bed with the arms crossed and resting on the knees. If in bed, the patient should lie on either the right or left side with the thighs flexed upon the abdomen, and the shoulders and head forward (Fig. 65) in order to give the maximum arch to the lumbar region; it is very important, however, not to have the shoulders or pelvis tilting or sagging as this very materially alters the direction of the needle.

Selecting the "Soft" Spot.—As stated above the proper spot for the puncture is exactly in the longitudinal middle line of the body between the third and fourth or between the fourth and fifth lumbar vertebræ. These are called the "soft" spots. By palpating the crests of the ilia and drawing an imaginary line or stretching a sterile towel between these points the "soft" spot between the fourth and fifth vertebræ may be located. As a general rule, the "spot" is just large enough to permit pressure by the index finger; the needle should be entered just a trifle below the middle of the "spot" and exactly in the middle longitudinal line.

Anesthesia.—A general anesthetic is not ordinarily required for adults unless the patient is very nervous and apprehensive when a

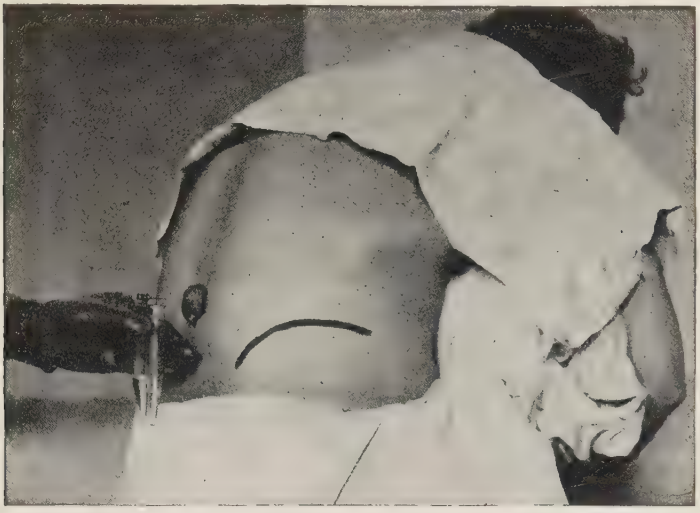

FIG. 64.—Spinal puncture in the sitting posture. The patient is an obese adult male, bent well forward over a folded pillow; the crests of the ilia are outlined. The needle has been passed between the third and fourth lumbar vertebræ in the median line; spinal fluid is being collected in sterile graduated tubes. (Keen's surgery.)

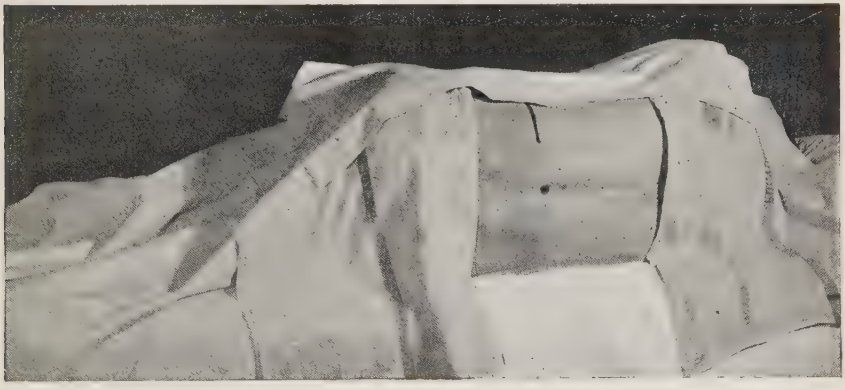

FIG. 65.—Proper arrangement for spinal puncture in the prone position. The back is well arched and perpendicular to the table. (From Kolmer's "Chemotherapy," W. B. Saunders Co., Publishers.)

few drops of chloroform or ether may be given principally for the psychic effect and rarely to the point of anesthesia, because local anesthesia of the skin, as about to be described, renders the opera-

tion almost painless. In tabes dorsalis and paresis the operation is peculiarly painless and even local anesthesia may be dispensed with in the case of those patients and particularly men who are not nervous or apprehensive; this always makes the puncture easier and more certain with the first passage of the needle, as infiltration of the skin and subcutaneous tissues with an anesthetic solution tends to obliterate the landmarks and sometimes deflects the needle.

For local anesthesia a sterile 1 per cent solution of butyn or novocaine may be injected superficially in the prepared skin of the "soft spot," until an elevated anemic spot is produced followed by reëntering the needle in a vertical direction in the exact middle of the spot and depositing a little of the solution as the needle is passed into its hub. Upon withdrawal, the spinal puncture needle is entered through the same hole in the skin.

Passage of the Needle.—The spinal puncture needle is now firmly grasped about 2 inches from its tip and passed in exactly the middle line directly inward by gentle pressure. After the skin has been passed, and with a perfectly sharp needle not larger than gauge 19 this is usually readily accomplished, whereas with a dull or large needle the skin is apt to wrinkle and deviate the needle from the midline, the needle is gently thrust forward slowly and deliberately, rather than quickly and with great force, until it suddenly meets with increased resistance followed by a "give away" sensation which to experienced fingers indicates that the subarachnoid space has been entered. Now, but not before, the stylet may be withdrawn and fluid collected. It is my practice usually to speak to the patient during the passage of the needle in order to conduct the operation in as gentle a manner and with as little pain as possible. I have no patience with or admiration for the almost brutal and abrupt manner with which spinal puncture is frequently conducted on fully conscious, apprehensive but cowed individuals.

If the needle meets with an obstruction it should not be forced as bone has probably been met with and its injury may result in a dull aching pain. The needle should be withdrawn partly or entirely and repassed.

Every effort should be made to pass the needle successfully with the first attempt and for this reason there should never be undue haste regardless of one's skill in conducting the operation. Every additional passage of the needle required before spinal fluid is secured only adds to the discomfort of the patient and greatly to the likelihood of post-puncture headache.

Measuring the Spinal Fluid Pressure.—The pressure should be measured before there is an escape of more than a few drops of fluid, but one must be sure that the flow is free of obstructions. The method of adjusting the instrument is shown in Fig. 63, page 454. As previously mentioned, the patient should be lying on the right

or left side, breathing easily and without coughing or other muscular movements. Even a deep breath may slightly increase the pressure, and violent excursions of the manometer results from coughing, change of position, etc.

The normal pressure with a mercury manometer is from 4 to 10 mm. with an average 6 to 8 mm.; a pressure of 12 mm. is border-line while 15 mm. or higher is distinctly increased. In children the normal pressure is about one-third less. If the pressure is taken in millimeters of water (normal 60 to 120) it may be expressed in millimeters of mercury by dividing by 13.

A rough gauge of the pressure may be estimated by the rate of flow of fluid from the needle after some experience has been gained with a needle of uniform bore; at least one is able to say whether or not the fluid pressure is distinctly increased or not, without being able to detect the finer differences.

The pressure is about one-third higher in the sitting posture, the upper limit of normal being about 14 mm. of mercury.

Collection of Fluid.—The fluid is now collected and for this purpose two sterile test-tubes should be employed. The first flow is collected in Tube No. 1 as it may be blood-tinged and unfit for cell counts and protein determinations but acceptable for the Wassermann test; about 2 cc. should be collected followed by 2 cc. in Tube No. 2 (for cell counts, protein tests and colloidal gold test) and lastly by an additional 1 or 2 cc. in Tube No. 1; this gives a total of from 5 to 6 cc. of fluid which is ample for all tests. If the fluid runs perfectly free of blood the first few drops may be discarded and about 5 cc. collected in a single test-tube or vial.

The pressure may be taken once or twice during the collection and especially in selected cases in whom a sudden or great drop in pressure is to be avoided. If headache or syncope develop the drainage should be stopped at once.

Dry Tap.—If spinal fluid fails to flow when it is believed that the needle is in the subarachnoid space, it is frequently designated as a "dry tap;" but occurring in cases of syphilis, it may be stated that almost invariably it is due to the fact that the needle is not actually in the subarachnoid space. The needle may be clogged with coagulated blood, a bit of tissue or the opening may be closed by contact with the arachnoid; it should be gently turned, pulled out or advanced very slightly. If these fail it should be withdrawn, inspected and cleaned if necessary and repassed. In rare cases of old meningitis with adhesions or tumors obstructing the subarachnoid space, "dry tap" or a very scanty flow of fluid may occur, but in the great majority of instances, "dry taps" in syphilis are due to errors or accidents of technique.

After-treatment.—The needle should be rapidly withdrawn, the iodine thoroughly removed with alcohol and the puncture sealed with sterile cotton and collodion.

As previously stated, the patient should remain in bed for at least eighteen to twenty-four hours as originally advised by Quincke.

If the puncture is done in the clinic or office, the patient should be advised to go home immediately, lie down and retire early. As a general rule, it is permissible to get up and resume usual duties next day, but if headache develops upon arising it is usually necessary to lie down again for relief.

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CHAPTER XL.

SPECIFICITY OF THE KOLMER COMPLEMENT-FIXATION REACTION IN SYPHILIS.

The matter of the *specificity* of the Wassermann reaction or complement-fixation in syphilis is unquestionably of most importance in relation to the serum diagnosis of the disease. The literature and particularly the early literature contains so many reports on the occurrence of falsely positive reactions in other diseases that the majority of physicians are under the impression that almost any condition is capable of yielding a falsely positive reaction. This is a serious mistake largely due to defective methods and errors in technique.

Even Bruck[1] in his recent book mentions scarlet fever, typhus fever, malaria, chancroid, acute lupus erythematosis, tumors and various other diseases as capable of uniformly or occasionally yielding falsely positive reactions but he could not escape doing this since so many different and defective methods are being employed. Since he has adequately reviewed the literature up to 1924 I shall refrain from doing so and largely confine myself to a brief discussion of the results observed by others and myself with my new antigen and method.

THE INFLUENCE OF TECHNIQUE UPON SPECIFICITY.

Biological Aspects.—Even a casual and superficial review of the literature shows at once that the results of studies upon specificity have been tremendously influenced by technique and it is to be regretted that so many writers omitted the essential details in their publications. Frambesia tropica or yaws seems to be the only disease in which there has been a general agreement on the occurrence of positive reactions and this is to be expected since it is caused by a very closely related organism (*Spirochæta pertenuis*) and indeed is regarded by some competent observers as a possible tropical form of syphilis.

As I shall shortly discuss with more detail my method does not yield falsely positive reactions in trypanosomiasis (experimental), leprosy, malaria, scarlet fever and other febrile and non-febrile diseases and conditions. This is particularly significant in leprosy, since nodular cases of this disease unquestionably tend to yield positive reactions and there must be an explanation of the results.

Apparently some obscure changes may occur in the blood serum in leprosy and during the febrile periods of malaria, typhus fever, scarlet fever, etc., which increases the tendency to non-specific reactions and particularly when 0.4 per cent cholesterolized extracts are employed as antigens; furthermore it is well known that the sera tend to be more anticomplementary than usual. In my method the influence of these serum changes is reduced to a minimum or entirely removed because the new antigen is prepared of beef heart, contains but 0.2 per cent cholesterol and is so free of anticomplementary activity that the usual dose of 10 antigenic units is from thirty to fifty times less than the anticomplementary unit. This, along with the daily titrations of complement and hemolysin, avoiding the use of defective complement serum and weak hemolysin, and a primary incubation at 6° to 10° C. instead of at 37° C. are the *biological* reasons for the specificity of the reaction for syphilis in those localities where yaws does not occur. On the other hand, when cholesterolized antigens and particularly those prepared of heterogenetic guinea-pig heart are employed in doses only four to ten times under their anticomplementary unit as is usual in other methods, the chances of securing pseudo-positive reactions is greatly increased and especially in leprosy.

From the *biological* standpoint therefore positive reactions cannot be avoided in frambesia because the antibody in this disease is practically identical with the syphilis antibody; possibly the same is true in relapsing fever since it is also caused by a spirochete (*Spirochæta recurrentis*). But in other diseases like leprosy of both types, malaria, trypanosomiasis, scarlet fever, typhus fever, pneumonia, diabetes, etc., the influence of the serum changes which *may* occur can be prevented by technical procedures as shown by uniformly negative reactions not only by my method but likewise by some other methods as well and notably that of Browning and McKenzie.

Technical Aspects.—But the chances for technical errors will always remain since every test is subject to it. None can be made error-proof regardless of safeguards and simplicity of technique. There is always the human equation. But they can be almost entirely removed by skill, experience and conscientious effort. There is no excuse for mislabelling specimens, for errors in making dilutions, using dirty glassware and defective reagents, undue haste, etc., and physicians should give up the pernicious habit of pressing laboratories for *quick* reports at the expense of accuracy and all laboratories should abandon foolish competition on the basis of quick service. What is worth doing at all is worth doing well and in no other branch of laboratory diagnosis is this dictum truer than in the serum diagnosis of syphilis. Enough harm has been done by insufficiently trained and inexperienced technicians to call a halt upon the practice of submitting the serum diagnosis of so important a disease to their unsupervised care. Any test conducted in a blind

manner by putting this and that in a test-tube in such and such amounts must come to grief sooner or later. The worker should appreciate the necessity of thoroughly understanding the reagents and be able to analyze difficulties and possible sources of error.

SPECIFICITY IN RELATION TO THE SENSITIVENESS OF THE COMPLEMENT-FIXATION REACTION.

I wish to make it perfectly plain that I have deliberately sacrificed some degree of sensitiveness in my method to gain specificity; in other words I have sought only the maximum of sensitiveness consistent with specificity. All experienced serologists know that by changing the hemolytic system and dose or kind of antigen that it is possible to make any method supersensitive or "over-delicate" but that these changes likewise increase the number of falsely positive reactions. For example this is easily possible in my method by using 1 instead of 2 full units of complement. But I would rather miss an occasional case of latent syphilis than obtain a falsely positive reaction in a single individual which may cause a great deal of mental suffering on the part of some before the error is corrected while indeed the damage may be irreparable. Some of my colleagues have criticized this attitude but it is a philosophical and practical subject upon which a difference of opinion can be readily held.

Therefore from the *diagnosis* standpoint "over-delicacy" or supersensitiveness is a serious defect for *routine* work. This does not mean, however, that the test should not be as sensitive as consistent with specificity. Not all cases of syphilis and especially when detected in elderly or debilitated individuals demand the institution of treatment; I well know that it is sometimes required to leave good enough alone but it does no harm for the *physician* at least to know that the patient *is* syphilitic. Furthermore I can appreciate the particular and peculiar circumstances surrounding the diagnosis of syphilis in the Army and Navy and why these laboratories require strongly positive reactions before making the diagnosis of syphilis since the matter of pension, etc., may be involved. But yet the aim should be the *maximum of sensitiveness consistent with specificity* and especially when the test is employed as a control on the efficacy of treatment in known cases of syphilis since it is to the interest of the patient that the slightest traces of the infection should be detected.

THE SIGNIFICANCE OF UNEXPECTED AND WEAKLY POSITIVE REACTIONS.

In relation to specificity and the practical serum diagnosis of syphilis no reactions are more important than the unexpected positives and especially the weakly positive complement-fixation and

precipitation reactions. In cases of known syphilis undergoing treatment weakly positive reactions ordinarily present no difficulties in interpretation; nor in cases of known syphilis in whom weakly positive reactions recur after previous negative reactions.

But in cases examined as part of a routine and in whom no clinical or historical evidences of syphilis are obtainable or in whom these are very indefinite, weakly positive reactions present the greatest difficulties in interpretation and place the greatest strain upon specificity. Are the reactions due to latent and unsuspected syphilis? Or are they due to technical error? Or are they due to some diseased state other than syphilis?

In the first place unexpectedly positive reactions will always occur. Whenever clinical and serological results are in 100 per cent agreement something is wrong. It is difficult to state who is the greatest nuisance—the clinician whose opinions or the serologist whose tests are infallible. But the facts are clear namely, (*a*) that the serum test will and does detect cases of syphilis and especially latent chronic infections which cannot be detected by the most expert clinical or historical examinations alone; (*b*) that expert clinical examination will detect some cases of syphilis and especially congenital infections giving falsely negative complement-fixation and precipitation reactions and (*c*) that the serum tests may give falsely positive reactions largely because of technical errors just as clinicians may give falsely positive opinions.

It is useless and senseless to claim that the complement-fixation and precipitation tests can be made so reliable that under *routine* conditions positive reactions *always* mean syphilis; as I shall discuss shortly occasional falsely positive reactions occur and always will occur just as falsely positive clinical opinions have and will continue to occur. But as previously stated, it is essential to distinguish between falsely positive serum reactions due to errors in technique and therefore *avoidable* and those due to biological changes in the serum and perhaps *unavoidable*, just as distinctions should be drawn between the opinions of the expert and the inexperienced in clinical syphilology.

With faulty methods and careless or inexperienced technicians weakly positive complement-fixation and precipitation reactions *may* be a menace and a nuisance in the same manner as the opinions of cock-sure or inexperienced clinicians. But even with acceptable methods in the hands of careful and experienced workers it is to be expected that in the course of routine work occasional falsely positive reactions *may* occur. These may be due to a confusion of specimens of blood in the laboratory and I have known a number to be the result of a mix-up in labelling specimens in clinics and the private offices of physicians. But aside from these obvious errors it is

possible for super-sensitive complement or a slight anticomplementary action of serum or antigen or both to result in weak falsely positive reactions even though the serum and antigen controls show complete but tardy hemolysis. Obviously the number of these reactions is reduced to a minimum in the hands of experienced serologists.

In my laboratory the incidence has been less than 0.1 per cent or less than 1 in every 1000 tests with serum and even less with spinal fluids. Therefore while an unexpected weakly positive reaction like 1 – – – – or ± – – – – does not establish the presence of syphilis in the *absence* of clinical and historical evidence, yet the result should not be brushed aside as entirely lacking in significance. *At least the test should be repeated before informing the patient of the results.* Stronger reactions like 3 – – – – or 3 1 – – – are more significant and strongly positive reactions like 441 – – have always occurred in syphilis in my experience even though the presence of the disease was at first unsuspected. With my method I believe that much harm can be done by ignoring these unsuspected reactions even though they are but weakly positive since they are so likely to occur during the chronic concealed (so-called latent) stages of the disease or after prolonged absence from specific medication; I plead for their repetition at least and if possible without informing the patient, so that if they do happen to be non-specific the individual may be spared the shock of a positive report. And the same refers to the Kahn and other precipitation tests; even though they are more likely to yield positive non-specific reactions yet they are frequently specific and should not be ignored without careful consideration.

THE BEARING OF THE INCIDENCE OF SYPHILIS UPON SPECIFICITY.

In any study of the specificity of the complement-fixation reaction in leprosy, malaria and other diseases it must be carefully kept in mind that some true positive reactions are to be expected because of concealed or latent syphilis. Of course the incidence of these varies greatly according to social status and localities, but in the great American middle class I believe that they may be expected in at least 2 per cent of cases. In the tropics the incidence of syphilis is apt to be much higher and has greatly complicated studies upon the occurrence of non-specific Wassermann reactions in leprosy and malaria. Therefore before a non-syphilitic disease can be regarded as yielding positive reactions the incidence must be above the average level of syphilis as determined by an adequate number of control cases taken from the same class of individuals.

THE QUESTION OF SPECIFICITY IN FEBRILE DISEASES.

Quite an extensive literature has accumulated indicating that pyrexial states may induce temporarily non-specific positive Wassermann reactions. But I am sure that much of it is based upon errors in technique as the following brief review will show. On the other hand some observers (Boas, Teissier and Lutembacher) assert that high fever in acute measles, etc., may cause temporarily *negative* reactions in paresis and secondary syphilis and I am far readier to accept this possibility in view of the clinical and serological results being observed at present in the treatment of syphilis and particularly of the central nervous system by Jauregg- von Wagner's method of malarial inoculation.

Typhus Fever, Typhoid Fever and Pneumonia.—Wassermann has stated that in no case should reliance be placed on a positive result obtained with serum from a patient who has had fever within a week previously. But I am sure that the importance of this has been greatly over-emphasized although it may be that fever can produce a change in the serum leading to an increased anticomplementary state of the serum and consequently to non-specific reactions; for this reason *weakly* positive reactions like 1 – – – – should be interpreted with extra caution in the absence of all clinical or historical confirmatory evidence.

Typhus fever especially has been stated by Bauer[2] to give falsely positive reactions in as high as 92 per cent of cases before the crisis but Papamarku[3] and other workers have recorded a proportion of positive results which can probably be accounted for by latent syphilis. In 6 cases of *typhoid fever* tested during the height of the fever I have observed completely negative reactions in all. In my opinion neither disease produces biologically positive reactions.

The same is true of *lobar* and *bronchopneumonia*. In a series of 13 cases Steinfield and myself[4] observed negative reactions in all except 1 syphilitic and since our study I have tested the sera of 24 additional cases of lobar pneumonia before and after the crises with uniformly negative reactions. I am quite sure that *pneumonia in non-syphilitic individuals* does not give positive reactions in my method.

Scarlet Fever.—*This disease is certainly to be dropped from the list said to be capable of yielding non-specific reactions.* It is difficult to explain why so many investigators have observed positive Wassermann reactions unless their antigens and general technique were responsible. In 1911 I found[5] only 2 per cent of positives out of 250 cases and none of a second series of 30 cases have reacted positively by my new method, sera being tested from the second day to the sixth week of the disease.

Tuberculosis.—Because of the wide prevalence of this disease the question of its producing falsely positive reactions is of considerable importance. A large literature indicates that the serum during high fever *may* give a false reaction with 0.4 per cent cholesterolized antigens, but I am quite sure that *these reactions do not occur with my antigen and new method*. At least Dr. Callahan and I observed none in a series of about 200 acute and chronic cases; some positive reactions occurred but only in those in whom syphilis was clinically present and Kilduffe[6] has had a similar experience. Boas and With[7] have recently stated that the chances of a tuberculous non-syphilitic person giving a positive reaction is not more than 1 in 1000 and whenever a positive reaction occurs it is necessary to suspect the coincident presence of syphilis.

Malaria.—During the febrile stages of malaria and especially when parasites are present in the blood, non-specific positive reactions are said to occur and to disappear under quinine medication. But there is the widest divergence of results and opinions in the large literature which has accumulated upon this subject. Some cases yielding positive reactions during life have shown evidences of syphilis after death, a striking case being recorded by McConnell;[8] in others the lesions of syphilis could not be detected after death. But the wide prevalence of syphilis in tropical countries has added greatly to the difficulties in arriving at reliable conclusions.

In a series of tests with specimens of sera kindly furnished by Dr. Bates of Ancon, Panama, and Dr. Caldwell of the Public Health Service in Houston, Texas, I found[9] the reactions uniformly negative with all but 2 patients in whom the possibility of syphilis could not be definitely excluded. Since then tests with the sera of 21 additional cases of tertian and æstivo-autumnal cases of malaria in individuals in whom the presence of syphilis could be excluded with a fair degree of accuracy have yielded uniformly negative reactions, and I am of the firm belief that the sera of non-syphilitic malarial patients do not yield falsely positive reactions in my new method even when blood is drawn just before, during or after a paroxysm of chills and fever. The occurrence of positive reactions in malaria is presumptive evidence of syphilis requiring very careful clinical study, and this opinion is shared by Iyengar,[9] Johnson,[10] Thomson and Mills[11] and other recent investigators. Furthermore it is of significance that the Wassermann reaction in paretics artificially inoculated with benign tertian malaria in treatment do not show *stronger* reactions even after ten or more paroxysms over several weeks of time sufficient for reagin production if such occurred; on the contrary it has been my experience with cases from the clinic of Dr. Schamberg that the quantitative serum reactions in my method have become progressively weaker, even reaching negativity in some cases. Under these conditions it has appeared to me very

improbable that malarial parasites *per se* could produce the reagins responsible for positive Wassermann reactions.

Vincent's Angina.—So far I have observed no positive reactions in Vincent's angina, although it is to be admitted that the possibility of their occurrence exists since a spirochete is involved in its etiology. During the past year one case extensively involving the fauces and yielding a 2 ++++ reaction in another laboratory has be brought to my attention. This test was not immediately repeated, but following recovery several repeat tests yielded negative reactions. On the whole I think it quite unlikely that ordinary Vincent's angina of the fauces including infections of the gums (*trench mouth* or spirofusillar gingivitis) are capable of yielding positive reactions.

Numerous cases of other febrile diseases in non-syphilitic individuals, embracing *ulcerative endocarditis*, *acute rheumatic fever*, *meningitis* (meningococcic, pneumococcic, streptococcic and tuberculous), *follicular tonsillitis*, *furunculosis*, *chancroid*, *gonorrhea* and various surgical diseases as *osteomyelitis*, *cholecystitis*, etc., in non-syphilitic individuals have uniformly yielded completely negative reactions. *Acute anterior poliomyelitis* likewise yields negative reactions as observed by Freese and myself[12] and the same is true of *lethargic encephalitis*.

THE QUESTION OF SPECIFICITY IN NON-FEBRILE DISEASES.

Leprosy.—Probably most interest is to be placed in the possibility of non-specific reactions in leprosy. Undoubtedly the serum in this disease and particularly from cases of well-advanced nodular leprosy may yield positive Wassermann reactions with most methods in common use, but in a study of the new complement-fixation test in this disease by Denney and myself[13] it was found that falsely positive reactions did not occur. Positive reactions were, of course, observed, but in these there was either historical or clinical evidence of syphilis, and I believe that the new test is not at all likely to yield non-specific positive reactions in pure leprosy, due primarily to the fact that the dose of antigen employed is thirty to fifty times less than its anticomplementary unit. With the old three-antigen method apparently falsely positive reactions occurred in at least 7 to 8 per cent of apparently non-syphilitic leprous patients in confirmation of numerous similar reports in the literature, but these were not observed with the new method (Table 74), and the Pineas[14] have had a similar experience, stating in conclusion to their extensive studies in complicated and uncomplicated cases of leprosy in the Culion Leper Colony, that the findings indicate that with Kolmer's and other refined methods the Wassermann reaction is negative in uncomplicated cases in their ordinary phase; also that when the reaction is clearly positive in ordinary cases that it has the same significance as in non-lepers.

TABLE 74.—COMPLEMENT-FIXATION REACTIONS BY THE NEW METHOD IN LEPROSY.*

Cases of leprosy.	Number tested.	New method.		Old method.	
		Positive.	Negative.	Positive.	Negative.
With no evidence of syphilis	125	None	100%	7.2%	92.8%
With evidence of syphilis .	27	100%	None	100%	None
Syphilis suspected . . .	7	None	100%	None	100%

* 159 cases: nodular, 39; anesthetic, 32; mixed, 88.

In a more recent study Denney, Barentine, Yagle and myself have observed that the specific treatment of syphilis occurring in lepers has an influence upon the reactions similar to that observed in non-leprous syphilitics; also that cowpox vaccination may reduce or entirely remove positive reactions for a month or two which is probably due to the influence of the fever and infection upon *Spirochæta pallida* as well as upon *Bacillus lepræ*.

Pregnancy.—The same is true with the sera of non-syphilitic pregnant women tested at or near term. In the experience of Smith[15] the new test does not yield falsely positive reactions in this condition and my experience has been exactly similar. When positive reactions have occurred they have remained positive after delivery in the majority of cases and have been observed only in syphilitic women. In this connection it is to be borne in mind that pregnancy may stimulate or provoke latent syphilis into activity just as trauma, intercurrent disease, the administration of arsphenamin, etc., may prove provocative so that unexpected positive reactions may occur, but when these are observed with the new method I believe that syphilis is present rather than that the reactions are falsely positive due to metabolic, toxic, or other changes in the serum in late pregnancy (eclampsia, etc.). Kilduffe[16] has had similar experience with 201 cases. Furthermore it would appear to be definitely established that the Wassermann reaction in pregnant syphilitic women may become negative for a time after delivery and this is doubtless responsible for much of the confusion. Dr. A. W. Stillians[17] of Chicago informs me that of 52 cases of pregnancy giving positive reactions by my method during pregnancy, 41 continued to do so after delivery while 11 reacted negatively, and all of whom were regarded clinically as syphilitic. In his whole series of 6954 pregnant women, mostly white, 6 per cent reacted positively; of 814 pregnant Negro women, 19.2 per cent gave positive reactions, practically all of whom were regarded as syphilitic. Certainly as stated by Stillians the estimate of Belding[18] that "as high as 25 per cent of the positives in pregnant women with cholesterolized antigens may prove non-syphilitic" is a gross exaggeration evidently due to faulty technique.

Diabetes Mellitus.—Recently Rockwood and Sanford[19] have shown in an excellent serological study of 501 cases of diabetes that falsely positive Wassermann reactions are of rare occurrence in this disease. Among these there were 8 cases of probable coincidental syphilis with apparently falsely negative reactions with the sera of 2 by a modified Noguchi method and 1 by my method; 5 yielded positive reactions but in 2 of these the evidences of syphilis were not conclusive. In 35 cases studied by Steinfield and myself[20] with blood sugars of 170 to 460 mg. per 100 cc. and several in coma, the reactions were negative and it is my firm conviction that positive reactions by the new method in diabetes and especially repeated positive reactions are always due to coincident syphilitic infection.

Nephritis and Uremia.—From time to time investigators have reported falsely positive Wassermann reactions with the sera of non-syphilitic individuals with acute and chronic nephritis with high retention and especially in uremic coma. These may occur with some methods but in a series of 17 cases examined by Steinfield and myself showing from 21 to 179 mg. of urea per 100 cc. of blood and several in uremic coma, completely negative reactions were observed in the new method. Since then a large number of additional cases have been tested in the medical clinics of the Graduate Hospital, Philadelphia, with similar results so that I believe nephritis and uremia in non-syphilitic individuals incapable of producing pseudo-reactions.

Jaundice.—Falsely positive Wassermann reactions have also been reported from time to time in individuals with jaundice. Lange[21] states that biliary acids in the blood may act as antigens and give positive Wassermann reactions especially in acute yellow atrophy of the liver whereas in pure bilirubinemia without cholacidemia which often occurs in hemolytic jaundice, this possibility is not present because of the absence of the acids.

I have had no experience with acute yellow atrophy but in cholecystitis, biliary cirrhosis and obstructive jaundice due to carcinoma of the pancreas occurring in non-syphilitic individuals, the reactions by my method have been uniformly negative in the experience of Steinfield and myself, and Rockwood and Beeler have had a similar experience. It is true that sera deeply tinged with bile products are to be carefully watched from the standpoint of anti-complementary reactions as already discussed in Chapter VII, but pseudo-positive reactions are readily avoided.

Anesthesia.—I have never observed positive reactions in non-syphilitic individuals after ether, chloroform and gas-oxygen anesthesia by my method, specimens being taken during and again immediately after recovery from the effects. Nor have I observed these anesthetics to give falsely negative reactions in a smaller series (12 cases) of syphilitics giving weakly to strongly positive reactions.

Thyroid Disease.—Nor have I observed positive reactions in cases of thyroid disease in non-syphilitic persons (6 cases of Basedow's disease and 11 of simple goiter). It has been stated that the serum in thyrogenous psychosis may give falsely positive reactions but I doubt if this is true of non-syphilitic individuals examined by acceptable methods.

In this connection brief mention may be made of the report by Mayer[22] stating that complement-fixation tests conducted with an antigen of iodothyron (*Dietrich reaction*) yield positive reactions during infectious and anaphylactic fevers as well as in hyperthermias due to over-heating; also in afebrile thyreotoxicosis but not in febrile athyreoses. It is believed to differentiate between fevers of nervous and infectious origin; I have had no experience with it.

Tumors.—During the past eight years a large number of cases of carcinoma, sarcoma and various non-malignant tumors have been tested by the new method without the slightest evidence of non-specific reactions even though the literature contains numerous references to alleged falsely positive Wassermann reactions by various methods and especially in tumors of the central nervous system. It is true that positive reactions are sometimes encountered in cases presenting tumors but so far these have occurred only in syphilitic individuals in some of whom the tumors themselves were gummata.

Lead Poisoning.—This is another disease sometimes mentioned as capable of yielding non-specific reactions. In 5 cases coming under my observation the reactions were completely negative in all but 1 who gave a history of infection ten years previously with a brief course of mercurial treatment. Gjorgjevitch and Piévatchevitch[23] have stated that intravenous injections of *iodoform* may cause positive Wassermann reactions in rabbits, although subcutaneous injections were without effect, and that the treatment of chancroids with this substance may be responsible for falsely positive reactions. In my experiments, however, rabbits did not produce positive reactions and I believe their results may have been due to the non-specific reactions so apt to occur with normal rabbit sera.

Kala-Azar.—Various forms of Leishmaniasis and particularly kala-azar have been stated to yield pseudo-positive reactions but the difficulties of excluding syphilis are obvious and the very careful work of Iyengar[24] has shown that the incidence in India among cases of this disease is practically the same as the incidence of syphilis (20 per cent); in his opinion the reactions in kala-azar are due to concealed or latent forms of the latter disease.

Negative reactions have also occurred routinely in cases of *psoriasis, eczema, lupus erythematosis, acne, lichen planus* and various other skin diseases among non-syphilitic individuals referred from the clinic of Dr. Schamberg. I have had no experience with *pellagra* and *beri-beri* but expect that the results are similar.

Arsphenamin Treatment.—It is well known that the administration of arsphenamin and its congeners may provoke positive Wassermann reactions in cases of concealed or latent syphilis yielding previous negative reactions. When such occur there is usually no reasons for suspecting their specificity but Strickler, Munson and Sidlick[25] have raised the question whether or not arsphenamin administered to non-syphilitic individuals is capable of producing changes responsible for temporary pseudo-positive reactions. Their studies were made with clinic patients presenting no obvious manifestations of syphilis but is apparent that the possibility of some of the positive reactors being cases of concealed syphilis could not be ruled out with certainty.

For this reason I conducted experiments with normal rabbits with entirely negative results[26] which were later confirmed by Kilduffe and Kissmeyer. Since then I have used arsphenamin and neoarsphenamin by intravenous injection in the treatment of severe cases of pernicious anemia and 3 of severe chloroanemia. None have yielded positive reactions by my new method before or after the administration of 2 to 8 doses and therefore I very much doubt if these organic arsenicals are capable of producing falsely positive reactions in non-syphilitic individuals.

THE QUESTION OF SPECIFICITY WITH POSTMORTEM SERA.

Since Bruck originally stated that sera obtained postmortem might react positively without existing syphilitic infection quite a controversial literature has accumulated.

Unquestionably putrid specimens and sera very deeply tinged with hemoglobin are particularly apt to be anticomplementary as is likewise true of cord bloods. Doubtless the occurrence of these explains a considerable number of alleged non-specific reactions and it should never be overlooked that a serum may give a completely hemolyzed control but yet be anticomplementary enough to give weak inhibitions of hemolysis in the presence of antigen. The particularly extensive and valuable investigations of Graves[27] have shown quite conclusively that sera obtained soon after death and before these postmortem changes have occurred yield almost identical results with antemortem tests and my own experience with the new method is in entire agreement with his results in the case of specimens obtained within twenty-four hours after death.

THE INFLUENCE OF ALCOHOL UPON SPECIFICITY.

Craig and Nichols[28] have stated that the Wassermann reaction may be rendered temporarily negative in syphilitics during or immediately after acute alcoholism (6 to 8 ounces of whisky taken in the course of twelve hours). Boas also has made a similar statement but Browning and McKenzie quote Dunlop to the effect that in

none of 14 cases was there any approach to conversion of the reaction from positive to negative under similar conditions.

I have never been able to study the question satisfactorily, that is, under the conditions described by Craig and Nichols, but in 8 cases of syphilis whom I was able to test during acute alcoholic debauches the reactions were positive in all.

Furthermore, with the assistance of Miss Rule I have tested daily 10 syphilitic rabbits yielding strongly positive reactions while they were kept deeply intoxicated by the frequent administration of 2 to 6 cc. of 50 per cent alcohol by stomach tube and roughly corresponding to 2 to 12 ounces of whisky per 60 kilograms of body weight. The majority of animals succumbed in three to five days but during this time there was not the slightest indication of any effect upon the complement-fixation reactions.

DISEASES OTHER THAN SYPHILIS WHICH MAY YIELD POSITIVE REACTIONS.

Unquestionably true positive Wassermann reactions occur in frambesia tropica or yaws, probably because the disease is caused by an organism (*Spirochæta pertenuis*) so closely related to *Spirochæta pallida*. Similar reactions also occur in gangosa of Guam and the Philippines (so called "tropical ulcerations") which has led Butler[29] and others to lean strongly to the idea that syphilis, yaws and gangosa are one and the same disease.

Positive reactions are also said to occur in *relapsing fever* but it has never been clearly established that positive reactions occur in *African sleeping sickness* produced by *Trypanosoma gambiense* and *Trypanosoma rhodesiense*. I have not been able to produce positive reactors among rabbits infected with *Trypanosoma equiperdum* and to the best of my knowledge the sera of horses suffering with *dourine* (*Trypanosoma equiperdum*) do not yield positive Wassermann reactions.

At all events these diseases are not confusing factors in most parts of the world and almost not at all in the United States, which leaves the complement-fixation test practically specific for syphilis under acceptable technical conditions.

THE SPECIFICITY OF SPINAL FLUID REACTIONS.

Of course much less investigation has been devoted to the question of the specificity of complement-fixation reactions with spinal fluids. My experience has covered the examination of fluids from all types of non-syphilitic meningitis (pneumococcic, streptococcic, staphylococcic, meningococcic, tuberculous and influenzal) *with negative reactions in all;* likewise in acute meningeal congestion or the so-called "serous meningitis" or meningismus of diabetic coma, uremic coma, lead poisoning, carbon monoxide poisoning, acute

alcoholism, pneumonia, measles and pertussis. Purulent spinal fluids are apt to be anticomplementary and may therefore yield non-specific reactions unless carefully tested but this technical error is easily avoidable.

The spinal fluid of 1 case of frambesia (yaws) gave strongly positive reactions in my experience; but this is to be expected and is the only disease definitely capable of yielding a biologically true Wassermann reaction. The spinal fluids of 3 lepers (all nodular cases) yielded negative reactions; likewise the spinal fluids of rabbits with chronic experimental trypanosomiasis (*Trypanosoma equiperdum*).

Stokes states that "non-specific reactions, usually partial, may occur in encephalitis (influenzal) and perhaps in rare cases of infectious meningitis and non-syphilitic brain tumor. Such non-specific positives must be conceded to be rare events, however." In 5 cases of lethargic encephalitis the fluids have been completely negative in my experience; also in 3 cases of non-syphilitic brain tumors.

The complement-fixation test with spinal fluid free of anticomplementary activity is therefore even more specific under proper technical conditions than in the case of sera and to the best of my knowledge is specific for syphilis when frambesia can be excluded, as is the case in the United States and most parts of the world; this does not mean however, that false reactions may not occur due to technical errors and especially weakly positive reactions.

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CHAPTER XLI.

SENSITIVENESS OF THE KOLMER COMPLEMENT-REACTION IN SYPHILIS.

As stated in the preceding chapter I have sought to make my test *as sensitive as consistent with specificity* in the serum diagnosis of syphilis and the question of its sensitiveness in the different stages of the disease and in treated cases now requires consideration.

COMPARATIVE SENSITIVENESS WITH METHODS EMPLOYING UNHEATED SERA.

Without doubt the heating of sera at 55° to 56° C. destroys complement-fixing antibody and for this reason I heat at 55° C. for fifteen minutes instead of for thirty minutes in order to reduce this destruction to a minimum (see Chapter XIX). But even under these conditions some destruction of antibody occurs and unheated sera would be preferred were it nor for the fact that they tend to give too many pseudo-positive reactions, in addition to the necessity of using perfectly fresh sera which is not always possible or convenient. But raw or unheated sera yield the most sensitive reactions and in my method I have sought to attain an equal degree of sensitiveness by other technical procedures and especially by using a new antigen and a primary incubation of sixteen to eighteen hours at 6° to 10° C. followed by ten to fifteen minutes in a water-bath at 38° C.

Comparative tests by Miss Rule, Miss Yagle and myself[1] with a group of carefully selected sera have shown that *the new method has proved equal or superior to raw serum tests in sensitiveness for the detection of syphilis antibody in the sera of syphilitic individuals under treatment and in clinically obscure cases of untreated syphilis.*

In this study the raw serum tests of Noguchi, Thompson, Bartlett and O'Shansky, Seelman and of Hecht-Weinberg-Gradwohl were employed with an antigen of acetone-insoluble lipoids; my test was conducted with the new antigen and sera in dose of 0.1 cc. as described in Chapter XXXI. The *percentage of true positive* reactions were as follows:

Noguchi	74 per cent
Thompson	82 per cent
Bartlett-O'Shansky	83 per cent
Hecht-Weinberg-Gradwohl	80 per cent
Seelman	78 per cent
New Test	86 per cent

COMPARATIVE SENSITIVENESS WITH METHODS EMPLOYING HEATED SERA.

In this investigation a comparative study was also made of the following tests employing heated sera: (1) The U. S. Army method of Craig employing an antihuman hemolytic system with cholesterolized alcoholic extract of heart muscle as antigen; (2) the method of Thomas and Ivy employing an antisheep system and an alcoholic extract of syphilitic liver as antigen; (3) the New York City Department of Health method employing an antisheep system and an alcoholic extract of guinea-pig heart as antigen and (4) my former or older method employing an antisheep system and three different antigens, one of which was a cholesterolized alcoholic extract of beef heart. The antigens were kindly furnished by the respective authors which added to the accuracy of the comparative tests and were carefully titrated for each method; the *percentage of true positive reactions* were as follows:

U. S. Army (Craig)	78 per cent
Thomas and Ivy	60 per cent
N. Y. City Department Health	60 per cent
Kolmer old test (cholesterolized antigen)	80 per cent
Kolmer new test	86 per cent

The sera were from treated and untreated cases of syphilis including non-syphilitic controls and the positive reactions by *all* methods were specific; the new method however, proved the most sensitive.

Miss Yagle and I have also compared the test with the U. S. Veterans' Bureau method[2] which includes several of the technical features of my test; 200 selected sera from syphilitic (treated and untreated) and non-syphilitic cases were examined simultaneously with both methods with the following results:

20 per cent positive by Veterans' Bureau method
26 per cent positive by Kolmer method.

In no instance was the Veterans' Bureau method positive and the Kolmer negative; all of the 6 per cent of cases giving positive Kolmer and negative Veterans' Bureau reactions were from cases of syphilis, the majority being under treatment in the clinic of Dr. J. F. Schamberg.

Possibly the best study of the sensitiveness of various methods including my own has been that conducted by Gilbert and Langworthy,[3] in 1926 under the auspices of the Committee on Standard Methods of the American Public Health Association.

Two hundred and fifty-two specimens from 227 patients were collected by Dr. M. B. Parounagian of the Bellevue Clinic in New York City and Dr. H. W. Potter, physician in charge of the State Institution for the Feeble Minded at Letchworth Village, and portions of each submitted to seven different laboratories listed below. Each laboratory was privileged to use its own technique. The sera

included 24 from primary, 6 from secondary, 24 from tertiary, 30 from congenital and 20 from cases of neurosyphilis; also 105 from cases of treated syphilis without present symptoms and 43 from non-syphilitic cases as controls.

The participating laboratories were as follows: (1) The New York Post-Graduate Medical School and Hospital, using the method of Smith and MacNeal[4] with the exception that two reaction tubes were employed instead of one, containing respectively 0.05 cc. and 0.02 cc. of serum, and a control tube containing twice the larger amount; (2) the Division of Laboratories of the Illinois State Department of Health; (3) the Western Reserve University School of Medicine, employing the method of Ecker;[5] (4) the Bureau of Laboratories of the Michigan State Department of Health, employing the Kahn test only; (5) the Wassermann Laboratory of the Massachusetts State Department of Health, employing the method of Hinton;[6] (6) my laboratory at the Hospital of the Graduate School of Medicine of the University of Pennsylvania, employing the method described in Chapter XXXI and (7) the Division of Laboratories and Research of the New York State Department of Health (Gilbert and Langworthy).

All sera were labelled with numbers only, so that none of the laboratories were acquainted with their source. All reports were sent to Dr. Gilbert, and the results were as follows on the 252 specimens including the controls:

TABLE 75.

Laboratory.	Per cent, positive.	Per cent, negative.	Per cent, unsatisfactory.
Post-Graduate Hospital, N. Y. (Smith and MacNeal)	38	61	1
Illinois State Department of Health (Hull)	25	74	1
Western Reserve University (Ecker)	32	58	10
Massachusetts State Department Health (Hinton)	16	83	1
Graduate Hospital, University of Pennsylvania (Kolmer)	45	54	1
New York State Department of Health (Gilbert and Langworthy)	58	39	3

As expected, the results varied considerably according to the antigens and primary incubation employed as shown in the results observed with the sera from the *syphilitic cases only* (untreated, treated and suspected):

TABLE 76.

Laboratory.	Antigens.	Incubation.	Per cent, positive.	Per cent, unsatisfactory.
Post-Graduate Hospital, N. Y.	4	Refrigerator 18 hours	28 to 66	0
Illinois Department of Health	2	Refrigerator 18 hours	30 to 31	0
Western Reserve University	2	Water-bath	27 to 31	11.0
Massachusetts Department Health	1	Water-bath	13.5	0
Graduate Hospital, University of Pennsylvania	1	Refrigerator 18 hours	50	0
N. Y. State Department of Health	3	Refrigerator and water-bath	39 to 62	2.4

Two of the laboratories employed my antigen in their methods but the results were inferior to those observed when the antigen

was used with my own method for which it was designed. Not infrequently my attention is drawn to the fact that *laboratories employing the Kolmer antigen in their method give physicians the impression that the Kolmer test is being employed, which is obviously unfair and has brought some discredit upon the method.*

It is also apparent that some of the methods employed in this investigation were strikingly inferior in sensitiveness, although all demonstrated an extremely high specificity for syphilis. Some were likewise unsatisfactory from the standpoint of yielding too many "unsatisfactory reactions," which meant that the hemolytic systems were too susceptible to the anticomplementary effects of serum.

Doubtless my method could be made still more sensitive by employing more cholesterol in the antigen, but I have preferred, at least for the present, to use but 0.2 per cent. Numerous reports by Schamberg and Klauder,[7] Schamberg and Greenbaum,[8] Smith,[9] Shivers,[10] Palmer and Gibbs,[11] Lynch,[12] Lorenz and Bleckwenn,[13] Kilduffe,[14] Kellogg and his associates,[15] Irvine and Stern,[16] Hartman and Reyner,[17] Harper and Curtis,[18] Maynard,[19] Thalhimer and Hogan,[20] Ottenberg and Wisler,[21] Sanford and his associates,[22] Cohen and Haythorn,[23] Kelly,[24] Becker,[25] Russell,[26] Greenbaum and Wright,[27] Johns and Jones,[28] Greenbaum and Yagle,[29] and others, totalling over 88,000 comparative tests with other methods, as recently summarized by Kilduffe,[30] have shown that it possesses a satisfactory degree of sensitiveness and practical specificity for syphilis.

COMPARATIVE SPECIFICITY AND SENSITIVENESS WITH PRECIPITATION REACTIONS.

During recent years various precipitation tests for syphilis have attracted considerable attention as substitutes for the Wassermann reaction, and particularly the Meinicke and Sachs-Georgi reactions in Germany, the Vernes in France, the sigma of Dreyer and Ward in England and the Kahn in the United States. As a general rule, their specificity and sensitiveness in the serum diagnosis of syphilis have been compared with the Wassermann reaction *without much regard to the technique of the latter.* For this reason some of the precipitation tests have been stated to be more specific and sensitive than the Wassermann reaction which may be true in view of the numerous complement-fixation methods of widely varying sensitiveness passing under the phrase "Wassermann reaction." *A closer study of them in relation to the serum diagnosis of syphilis would be much more serviceable than mere comparison with the Wassermann reaction* because the technical simplicity of the precipitation tests as a substitute for the complement-fixation reaction has been much over-rated, even though the former are of particular value on board ships, where it may be difficult or impossible to obtain sheep blood and complement for conducting the latter.

Insofar as the Sachs-Georgi, Meinicke (third modification) and Vernes (direct and indirect) reactions are concerned it would appear from the study made by Strumia[31] that 80 to 95 per cent of sera or an average of 90 per cent agree in results with my test but the percentage of agreement between the Kahn test[32] as now conducted and mine is much higher being in the neighborhood of 94 to 96 per cent in my laboratory under routine conditions. Under special conditions embracing the frequent retesting of those sera yielding discrepitant results as conducted by Kelly, the percentage of disagreements may be further reduced.

In the comparative tests with sera sent out by Gilbert and Langworthy referred to above the *percentage of positive reactions* in the different stages of syphilis in Kahn's laboratory (precipitation) and mine (complement-fixation) were as follows:

TABLE 77.

Stage.	Cases.	Kahn.	Kolmer.
Primary syphilis	24	58	67
Secondary syphilis	6	100	100
Tertiary syphilis	24	54	71
Neurosyphilis	20	80	70
Congenital syphilis	30	37	40
Treated syphilis	105	47	44

In other words under *routine conditions my test was generally more sensitive in untreated syphilis but the Kahn test slightly more sensitive in some cases of treated syphilis* which agrees entirely with the experience of Miss Yagle and me during the past four years, since both have been used routinely in the Laboratories of the Graduate School of Medicine of the University of Pennsylvania.

In my laboratory the sensitiveness and specificity of the Kahn reaction has been influenced by *the time when the readings were made.* When the tests were read after a fifteen minute incubation period in the water-bath at 37° C. following the usual two minutes of shaking and prior to the addition of saline solution, the percentage of true positive reactions in 207 tests was approximately 4 to 6 per cent *less* than observed in my reaction. On the other hand the Kahn reactions read after standing in the refrigerator over night at 6° to 10° C. were slightly more sensitive and about 96 to 97 per cent agreed with my reactions. The difference of 2 to 4 per cent was made up of sera reacting positively in the Kahn and negatively in the Kolmer or the reverse.

On the other hand, however, *when the Kahn reactions are read after an over night incubation in the refrigerator to secure the maximum of sensitiveness some non-specific reactions result* and especially weakly positive reactions like: —1 or—12. These have occurred with sufficient frequency in my practice (4 to 8 per cent) to make me hesitate accepting them when my test yields a completely negative reaction unless the patient is a known case of syphilis under treatment.

Furthermore, *the Kahn reaction is subject to as much variation in different laboratories as the complement-fixation reaction* despite its alleged greater simplicity which is commonly and erroneously thought to increase uniformity in results. This is probably due in large part to the difficulty of reading the weakly positive reactions. Of the sera sent out by Dr. Gilbert and Miss Langworthy in the study referred to above, Dr. Kahn reported 43.6 per cent positive reactions while the Kahn test in the Illinois State Department of Health yielded 31.7 per cent and in the Western Reserve University 51.2 per cent positive reactions, a variation of 7 to 12 per cent as shown in Table 78.

TABLE 78.—RESULTS OF PRECIPITATION TESTS IN TREATED, UNTREATED AND UNSUSPECTED CASES OF SYPHILIS.

Laboratory.	Test.	Percentage reactions.		
		Positive.	Negative.	Unsatisfactory or not tested.
Illinois State Department of Health	Kahn	31.7	68.3	0
Western Reserve University . .	Kahn	51.2	34.9	13.9
Western Reserve University . .	Kline	67.4	31.7	0.9
Michigan State Department of Health	Kahn	43.6	44.0	0.4

The non-syphilitic controls also showed some non-specific reactions (6.9 per cent) in one of the examining laboratories as shown in Table 79 and especially the microscopic test (27.9 per cent) of Kline and Young[33] although I believe that Dr. Kline has more recently improved his technique by stabilizing the antigen against false precipitation.

TABLE 79.—RESULTS OF PRECIPITATION TESTS IN CONTROLS.

Laboratory.	Test.	Percentage reactions.		
		Positive.	Negative.	Unsatisfactory or not tested.
Illinois State Department of Health	Kahn	0	100.0	0
Western Reserve University . .	Kahn	6.9	69.7	23.4
Western Reserve University . .	Kline	27.9	72.1	0
Michigan State Department of Health	Kahn	0	100.0	0

In summarizing their report Dr. Gilbert and Miss Langworthy state: "As there are sources of error in the performance of complement-fixation tests, leading to lack of uniformity in the results, it will be interesting to consider the results of precipitation tests made

by four of the laboratories on the same specimens. The precipitation test recommended by Dr. Kahn was performed in three of the coöperating laboratories. Two of these agreed fairly closely in the matter of marked reactions, but one failed completely to detect the partial and slight reactions. The test as performed in the third laboratory appears to err somewhat on the side of non-specificity, as 1 marked 1 partial, and 1 slight reaction were obtained with specimens from control cases. The slide precipitation test recommended by Dr. Kline, while showing the highest proportion of reactions, appears to be definitely non-specific, as reactions were obtained in 12 control cases, 5 of them being marked, 4 partial, and 3 slight. The precipitation test in Dr. Kahn's laboratory and in the other two laboratories using it showed marked differences in the results. In the case of one laboratory, there were 57 specimens and in the case of the other 52 specimens where the result of the test differed from those made by Dr. Kahn. In the former laboratory, 20 and in the latter 24 of the differences were between marked precipitation and no precipitation. This striking lack of uniformity in a test where identical technique was used far exceeds the variation in the results of complement-fixation tests obtained with the same technique. The precipitation test also showed variations in results of tests on successive specimens."

Dr. Davis Stern of the Division of Venereal Diseases of the Department of Health of Minnesota, has been employing both tests for several years and a recent summary of his results[34] has summarized the situation very clearly even though the figures in relation to the Kahn test are not by any means in agreement with some published reports. Of 58,000 tests 54,868 or 94.6 per cent were in agreement; 3132 or 5.4 per cent did not agree.

Of the latter 1897 yielded positive Kolmer and negative Kahn reactions: 1864 of these were cases of syphilis under treatment; 21 gave a history of syphilis; 3 gave no history or clinical signs and 9 were in process of study.

Six hundred and eighty-nine gave doubtfully positive Kolmer and negative Kahn reactions. Of these 2 showed primary lesions; 8 were congenital syphilis; 27 neurosyphilis; 2 cases of syphilis (stage not given); 1 was tuberculous and 649 were syphilitics under treatment.

Twenty yielded doubtful Kolmer and positive Kahn reactions. Of these 4 gave no history or clinical signs of syphilis; 2 were tuberculous and 14 were under treatment.

Five hundred and twenty-six yielded negative Kolmer and positive Kahn reactions. Of these 112 were pregnant women with no evidences of syphilis; 3 were cases of syphilis under treatment; 42 were tuberculous and 369 cases of general examination in whom syphilis was excluded.

In other words my complement-fixation test was more sensitive and just as specific as the Kahn test and the results are a clear example of how judgment may be influenced according to the manner or method of conducting the Wassermann test.

But these and thousands of comparative tests in my laboratory have shown that both the Kahn test and the complement-fixation test employing cholesterolized antigen and ice-box primary fixation are extremely sensitive and specific tests for syphilis and in my opinion *the serum diagnosis of the disease is best served by using both routinely* as has been my practice during the past four years. This is also the conclusion reached by Roderick, Salisbury and Cates,[35] based upon a study of both tests in 89 cases of syphilis receiving one to seven courses of treatment with the following results:

Kolmer positive and Kahn negative in 37 or 41.5 per cent; Kahn positive and Kolmer negative in 5 or 5.6 per cent; both positive in 30 or 33.7 per cent; both negative in 17 or 19.1 per cent.

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CHAPTER XLII.

CLINICAL INTERPRETATION AND PRACTICAL VALUE OF THE KOLMER COMPLEMENT-FIXATION TEST IN THE DIAGNOSIS AND TREATMENT OF SYPHILIS.

While my chief interest in connection with the serology of syphilis has been to render the technique of the complement-fixation test of increased specific sensitiveness and while I am able to point out in this chapter its great value in the diagnosis and treatment of the disease, yet clinical experience has likewise taught me its limitations and the necessity for evaluating the reactions in terms of combined serological and clinical experience. As a matter of fact we are able to evaluate the complement-fixation test *accurately* only in acute early syphilis where the clinical signs are definite and the spirochetes may be found; in latent or concealed syphilis and in the multitude of tertiary forms our best estimates can be only *approximately* correct because *clinical and roentgenological signs and opinions upon which evaluation is based are subject to as much variations as the Wassermann test in different laboratories*, and histories are so lacking in accuracy as to be frequently worthless.

Furthermore, the technique of the complement-fixation test has so much influence upon the results that I shall not attempt to review the subject of the clinical value of the Wassermann reaction in general but confine myself almost entirely to the results reported by others and observed by myself with the new method.

THE CLINICAL VALUE OF A POSITIVE BLOOD COMPLEMENT-FIXATION REACTION.

The new method has been found to possess such a high degree of specificity for syphilis as discussed in Chapter XL that there is no escape from the conclusion that a positive reaction carries at once very strong presumptive evidence of the presence of syphilis even though the disease is at first unsuspected. The only drawback to its absolute worth is the chances of technical error which varies with the skill, experience and carefulness of the serologist. Even weakly positive reactions should not be ignored but at least repeated. The number of disasters directly attributable to the almost unexplainable discard of positive reactions by physicians simply because they *thought* that syphilis could not be present, has been amply testified to by Stokes and other syphilologists.

Strongly positive reactions like 431 – – are particularly significant and false reactions due to errors in technique are rare in the hands of competent workers providing no mistake has occurred in the labelling of specimens.

I do not mean to infer that the diagnosis of syphilis should be made on the basis of a *single* positive reaction in absence of definite clinical evidence; but when the test is repeated with essentially similar results the evidence is quite conclusive even though the reactions are but weakly or moderately positive like 31 – – – or 2 – – – –. Certainly this has been my own clinical experience and the burden of proof rests upon the clinician to *prove* that syphilis is absent rather than upon the serologist to prove that it is present. After all, positive complement-fixation and precipitation reactions are but serological evidences of syphilis but under proper conditions they are the *best single signs of the disease and especially when repeatedly positive after the primary stage,* as amply verified by experienced syphilologists; only the uninformed, inexperienced and cock-sure clinician is apt to ignore the tests as valuable aids in diagnosis.

Furthermore, while positive serum reactions show that the patient is syphilitic they do not necessarily show that a *particular* lesion is syphilitic. Syphilis does not confer an immunity to other diseases and its victims are just as subject to cancer, tuberculosis and other diseases as non-syphilitic individuals. Therefore failure of a lesion to respond to a therapeutic test does not necessarily prove that the blood reactions were false.

THE CLINICAL VALUE OF A NEGATIVE BLOOD COMPLEMENT-FIXATION REACTION.

Unquestionably the margin of error is much more on the side of falsely negative reactions. The method is not too sensitive; rather it is not sensitive enough and this applies equally well to all other complement-fixation and precipitation tests and especially in the case of syphilis of women and children.

I believe that the strength or degree of the complement-fixation and precipitation reactions is in proportion to the degree of spirochetic activity even though this may not bear a relation to the clinical aspects because signs and symptoms may depend more upon *where* the organisms are located than upon the numbers present. Therefore relatively few organisms may produce signs of syphilis while too few to produce sufficient antibody for detection in the test-tube; this is especially true in tabes dorsalis, syphilis of child-bearing women and late congenital infections of children. Just how much spirochetic activity is required to produce enough antibody for positive reactions is not known and probably never will be; but it is apparent that the detection of small amounts varies

with technique and that it may be impossible ever to render any serum test sufficiently sensitive for the detection of *all* extremely latent infections although the *provocative* procedure still further reduces the incidence of falsely negative reactions.

The important lesson to be learned by the majority of practitioners is not to ignore positive reactions but to distrust negative reactions and never to accept a single negative as excluding syphilis when suspicious clinical signs or history are present.

On the other hand, a negative and especially two or more negative reactions exclude syphilis as a cause of *active* (progressive) lesions in over 90 per cent of cases. A quiescent syphilitic lesion may produce too little antibody for detection but an active lesion is very unlikely to do so unless very small in size. Therefore negative serum reactions are much more reliable in excluding syphilis as the cause of active lesions than of latent or quiescent ones. That is to say if a progressive lesion or lesions *may* be syphilitic, negative complement-fixation reactions carry great weight in excluding this possibility. It is true that the reactions may be negative at least temporarily even in secondary syphilis and especially that of pregnant women and likewise in the vascular types of neurosyphilis, but as I shall point out later at least 90 per cent of cases of syphilis after the primary stage show positive complement-fixation reactions if active or progressive lesions are present.

Daily Variations in Kolmer and Kahn Reactions.—Of further importance in relation to the danger of excluding syphilis on the basis of a *single* negative reaction is the fact that the antibody responsible for the complement-fixation and precipitation reactions is known to fluctuate almost daily. Craig[1] pointed this out fourteen years ago and several investigators have since corroborated his findings. Recently Greenbaum and Yagle[2] found in my laboratory that the sera of 8 cases of untreated syphilis in a series of 50 (16 per cent) gave daily variations in the Kolmer and Kahn reactions which went from one extreme to the other, although curiously enough when the Kahn gave a false negative the Kolmer was positive or the reverse in 6 out of these 8 cases. This demonstrated the advisability of conducting both tests on all sera; that variations in the antibody may occur from day to day; that variations may be also due in part to technique and lastly, that a single negative reaction may occur in untreated syphilis which is caught up by repeat tests.

THE CLINICAL VALUE OF ROUTINE BLOOD TESTS.

Since syphilis may attack almost every organ of the body it is a disease that concerns every specialty in the practice of medicine and surgery. But it is impossible to express accurately the incidence of the disease and therefore its clinical expectancy in practice.

Incidence based upon Wassermann surveys is too low and the intensive clinical studies of expert syphilologists have been applied to too few groups to give much aid. But it is known to vary greatly according to the social status of the group investigated; it is higher among the colored than among whites and higher among both in cities than in rural districts. It varies also according to the specialty being practiced and it has been conservatively estimated that from 1 to 30 per cent or more of the population are infected, with a general average of 6 to 10 per cent.

This is high enough to warrant and indeed demand the routine serological examination of *all dispensary and ward patients* and to include it in the complete examination of the majority of private patients. A great deal of the time devoted in hospital laboratories to useless routine urine and general blood examinations could be much better spent in conducting routine complement-fixation and precipitation tests on all cases including those occupying private rooms.

The frequency of syphilis of the bones and joints, liver and biliary passages, as well as the percentage of useless operations for gastric ulcer, gall stones, appendicitis and salpingitis among individuals whose symptoms are really due to syphilis and especially tabes dorsalis, renders the test an almost obligatory routine for the surgeon and especially the abdominal surgeon and orthopedist. Furthermore, it would reduce the incidence of extra-genital syphilis among surgeons and especially syphilis of the fingers following tonsillectomies.

Curiously the serum tests are apparently not employed as routinely by genito-urinary surgeons as the nature of their work would seem to demand. Doubtless this is due in large part to the use of dark-field microscopy in the diagnosis of chancres and to the confidence that many feel in their clinical judgment in the diagnosis and treatment of the disease in all of its stages; but the blood tests are almost indispensable in the diagnosis of chancres after the second week, in all atypical genital infections and certainly in all stages of the disease after the primary stage.

The frequency of cardiovascular, hepatic, gastric, pulmonary and other types of chronic syphilis masquerading as various clinical entities places the same obligation upon the internist including the gastro-enterologist and cardiologist.

Ophthalmologists in my experience are, as a rule, very skilful in the clinical detection of the pupillary, fundus and muscular changes of acquired and congenital syphilis, but grievous errors could be prevented by the more frequent use of the serum tests and especially examinations of the spinal fluid. And I am positive that rhinologists, laryngologists and otologists as a group too frequently regard syphilitic throats as Vincent's angina, gummas as benign

or malignant tumors, tuberculous laryngeal ulcers as syphilitic, as well as missing eighth nerve lesions altogether, to make it profitable for them to neglect the serum tests in a large proportion of their private and dispensary patients.

The high incidence of neurosyphilis, including approximately 20 per cent of syphilis among individuals with various mental disorders, renders routine blood and spinal fluid examinations almost obligatory for neurologists and psychiatrists in dispensary and in a high percentage of private patients.

The frequency of cutaneous and mucous membrane lesions in syphilis, as well as the multitude of chances of mistaking the various forms of seborrheic dermatitis, lichen planus, psoriasis, pityriasis rosea, papulo-necrotic tuberculids, erythema multiforme, ringworm and what not for the macular, papular, annular and follicular lesions of secondary syphilis, renders the tests of very frequent value in diagnosis and almost a routine for dermatologists until at least considerable clinical experience has been gained in differential diagnosis.

And the high incidence of congenital syphilis especially in dispensary practice has rendered the test a routine procedure in many institutions. I know of no more difficult field in the practice of syphilology than congenital and conjugal syphilis and certainly the diagnosis of late or tardy congenital syphilis offers many difficult problems calling for the coöperation of obstetrician, pediatrician, roentgenologist and serologist. As stated by Stokes[3] "in public and dispensary maternity practice there can be no question that with a 10 to 15 per cent incidence of positive results, the tests should be routine on all patients and should be repeated at least once or twice toward the middle and end of pregnancy. In private practice the physician may feel that his knowledge of the patient and family should have some weight. There can be no doubt, however, that the unobtrusive habitual performance of the test would forestall tragedies even in high places—a single weak positive or single negative with a suspicious background should never be totally disregarded. Observation and later tests of mother and child should follow all doubtful reports."

The discomfort attending the taking of blood for serum tests is so negligible as not to require discussion. The cost, however, constitutes an important item and especially for private patients. Every hospital laboratory, however, can afford to do the serum tests routinely; private room patients should be charged a moderate fee and it is easily possible for hospital laboratories to be self-supporting with efficient personel and equipment. And the fees charged patients in private practice even for routine tests represents a wise investment in the great majority of cases, while serologists as a group are just as ready to render their services gratuitously to the needy poor in dispensary and private practice as are clinicians.

CLINICAL VALUE OF ROUTINE SPINAL FLUID TESTS.

Owing to the greater inconvenience attending spinal puncture the routine examination of spinal fluids cannot and need not be as frequent as routine blood tests. But every case of syphilis requires at least one or two complete examinations of the fluid for total cells, protein, complement-fixation and colloidal gold or mastic reactions for diagnostic purposes and as a check on the results of treatment. All cases of primary and secondary syphilis should have a routine examination of this kind after a thorough course of treatment to determine whether or not infection of the central nervous system is present since the results influence the balance of the therapeutic program. And no study of latent or tertiary syphilis including relapsing infections can even begin to be complete without a thorough examination of the fluid, and especially since anywhere from 10 to 25 per cent may show negative blood reactions and positive spinal fluid changes. Furthermore, in latent cases infection of the central nervous system may readily escape detection by the most expert neurologists. There may be some clinicians who believe that spinal fluid changes in asymptomatic cases do not warrant or demand treatment but this is certainly contrary to my own experience and opinion and Stokes, Moore, Fordyce, Rosen, Schamberg, Soloman, Klauder and numerous other syphilologists emphasize the clinical importance of what Stokes has called "paper syphilis," that is, the spinal fluid changes of asymptomatic early and late neurosyphilis.

THE COMPLEMENT-FIXATION REACTION IN THE DIAGNOSIS OF PRIMARY SYPHILIS.

As Stokes has well written, the diagnosis of the chancre is no longer a clinical but a laboratory problem. So many chancres vary from the typical or classical description that even the expert may make mistakes and curiously enough the most experienced are usually the ones most likely to use the dark-field microscopic and serological tests as aids in diagnosis.

During the first seven days of the sore diagnosis is best made by dark-field microscopic examination of the secretion for *Spirochæta pallida* since 80 to 90 per cent are positive. However, local treatment may result in negative findings (reduced to about 30 per cent positive results) unless material is secured from the depths of the lesion and especially by aspiration. A heavy secondary infection and especially a superimposed chancroid infection may likewise mask an underlying syphilitic infection both clinically and microscopically. After the first week when adenopathy develops diagnosis may be made by gland puncture, the injection and withdrawal of a few drops of saline solution and examination for the organisms;

this is especially useful after the second week when healing of the chancre greatly reduces the chances of finding spirochetes on the surface.

But microscopic examination of lesions on the lips or within the mouth (tongue, gums and tonsils) must be conducted with great care since *Spirochæta microdentium* may be mistaken for *Spirochæta pallida*. In rare instances the chancre may be located within the urethra and demand repeated complement-fixation tests if the urethritis runs an atypical course. In women the chancre may not be detected at all since they are apt to be small and atypical and on the cervix easily mistaken for erosions.

Time is required for the production of the antibody giving positive complement-fixation and precipitation reactions. I have never observed a positive *blood* reaction by my method earlier than the fifth day after the appearance of the sore. Positive reactions by other methods have been reported as occurring on the first and second days but I surmise that these were recurrent chancres (monorecidives or chancre redux). Irvine has observed 75 per cent of cases giving positive blood reactions by my method in five to fourteen days after the appearance of the sores and the percentages of positive reactions in my experience have been as follows:

Five to fourteen days	44 per cent
Ten to fourteen days	75 per cent
Three to four weeks	82 per cent
Four to six weeks	94 per cent

Therefore the blood test is of value in practically all cases and *especially in those giving negative dark-field examinations and all suspicious oral, pharyngeal and tonsillar sores.* If negative reactions are observed the test should be repeated every three to five days for at least six weeks. As stated by Stokes "*Wassermann follow-up deserves the widest application in the management of all types of venereal disease and of every lesion, genital or extra-genital, which has aroused the slightest suspicion. Diagnostic knowledge has progressed far enough now to make it absolutely unjustifiable for any physician to give his patient a diagnosis of chancroid until four months have elapsed from the time of appearance of the lesions. Suspended judgment during this time would increase the amount of early syphilis recognized from 15 to 50 per cent. The Wassermann follow-up should also be applied to all patients with gonorrhea even in the absence of a genital sore. Systematic adoption of this procedure would identify at an early stage 16 per cent of syphilitic infections in which no history of onset other than that of gonorrhea can be obtained. Systematic application of Wassermann follow-up lesions in the mouth and throat would, without question, quite transform our conceptions of the extra-genital onset of syphilis and would nip many a tragedy in the bud*" (italics mine).

Complement-fixation Reactions with Chancre Secretions; the "Local" Wassermann Reaction. In 1922 Klauder and myself[4] reported that Wassermann tests conducted with *chancre secretions* and my former three antigen method gave 86 per cent positive reactions in 14 cases presenting chancres of three days to five weeks' duration while 57 per cent gave positive blood reactions. The material was collected after the method described on page 453.

Since then Stern and Rypins[5] have reported 100 per cent positive reactions with the method in a series of chancre cases showing 95 per cent positive dark fields and but 30 per cent positive blood reactions. Fuentes[6] has had a similar experience. Bragin[7] in a study of 62 chancres states that the reaction is specific and appears seven to fourteen days earlier than the blood reactions. Kitchévatz[8] has not met with as good results in a study of 215 cases but May and Prunell[9] in a study of 107 cases observed positive reactions in 43 per cent, positive blood reactions in but 8 per cent and positive dark fields in but 25 per cent.

Local treatment of the chancre has no effect upon the results and *in cases presenting negative dark fields and especially when facilities are not available for making these microscopic examinations it would appear well worth while to collect* (*technique on page* 453) *and submit chancre secretion as well as blood for the complement-fixation tests.* In a series of 6 cases of chancres (5 genital and 1 lip) of three to ten days' duration in my own practice the reactions with chancre secretions as tested according to the method described in Chapter XXXVIII gave positive reactions in 5 (83 per cent) and positive blood reactions in 3 or 50 per cent. The dark fields were positive in 5 or 83 per cent but the 1 case (a six-day genital lesion treated with local applications of 1 to 1000 bichloride of mercury) giving a negative dark field gave a positive "local" and a negative blood reaction.

THE COMPLEMENT–FIXATION REACTION IN THE DIAGNOSIS OF SECONDARY SYPHILIS.

Contrary to common impressions among practitioners the clinical diagnosis of syphilis in its secondary stage is not as easy in many cases as surmised and as stated by Stokes its "diagnosis has become practically entirely a laboratory issue."

With my method almost 100 per cent give positive blood reactions. During the past six years 2 cases have been brought to my attention giving negative reactions on the *first* test but all reacted positively when the tests were repeated. Moore has reported 3 negative reactors in a group of 32 secondary cases among pregnant women but among men and non-pregnant women the percentage of positive reactions on first tests is approximately 98 to 99 per cent and with repeat tests at least 99 to 100 per cent. Stokes states that repeated *negative* reactions excludes all but 1 per cent of cases.

As high as 60 per cent of patients found to have syphilis in later life can give no history of secondary lesions of any description and 24 per cent give no history of a chancre; only 82 per cent present the typical macular or maculo-papular rashes (Stokes). The same author states that constitutional symptoms do not have a high diagnostic value since they occur in but 63 per cent of men and 42 per cent of women, while adenopathy is detectable in but 70 per cent and splenic enlargement in but 30 per cent.

That the clinical diagnosis may be "fugitive and inconspicuous" is well borne out by the numerous diseases listed by Stokes as capable of mistaken diagnosis for syphilis as follows:

(*a*) Macular and papular syphilids mistaken for
- Seborrheic dermatitis
- Pityriasis rosea
- Leprosy
- Measles
- Toxic erythemas
- Psoriasis
- Small-pox
- Lichen planus
- Papulonecrotic tuberculids
- Chronic urticarias

(*b*) Annular syphilids for the annular lesions of
- Ringworm
- Seborrheic dermatitis
- Impetigo contagiosa
- Pityriasis rosea
- Lichen planus
- Erythema multiforme

(*c*) Follicular syphilids for
- Chronic urticaria
- Lichen scrofulosorum
- Pityriasis rubra pilaris

(*d*) Syphilitic alopecia for alopecia areola.

Furthermore not all condylomata are syphilitic although the flat lesions usually are while condylomata accuminata usually are not. Dark-field examinations are valuable in differential diagnosis as well as in the diagnosis of mucous patches but as stated above it is impossible (at least in my experience) to differentiate microscopically between *Spirochæta pallida* of syphilis and *Spirochæta microdentium* of the mouth. Furthermore according to Stokes mucous patches may be mistaken clinically for:

Vincent's angina
Diphtheria
Streptococcus sore throat
Carcinoma of the tonsil
The bullous lesions of erythema multiforme
Malignant pemphigus
Oral and genital aphthæ
Oral and genital lichen planus
Marginal and exfoliative glossitis
Mercurial and bismuth stomatitis
Herpes progenitalis
Scabies

None of these diseases are capable of giving *biologically* pseudo-positive reactions in my method which renders it practically specific for the diagnosis of 99 to 100 per cent of cases of secondary syphilis.

THE COMPLEMENT-FIXATION REACTION IN THE DIAGNOSIS OF LATENT (CONCEALED SYPHILIS.)

But in the diagnosis of latent or concealed syphilis the complement-fixation and precipitation tests have their greatest value. A history of chancre and secondary lesions is so frequently lacking as to be very misleading and clinical diagnosis may escape even the most expert, especially in the acquired syphilis of women and late congenital syphilis of children.

I refer to that large group of cases usually seen from a few to as many as forty or more years after infection, with or without previous treatment, which are detected in the course of routine examinations or as a result of consulting the physician for some non-syphilitic or atypical syphilitic complaint. The disease is "latent" only in the sense of presenting non-obvious or detectable lesions of syphilis but not usually truly latent in the sense of inactivity on the part of the spirochetes since these are always apt to be active in the production of concealed lesions. Obviously the percentage of clinical detection varies with clinical skill and experience just as serological detection varies with the specific sensitiveness of the method, skill and experience of the serologist.

Among all cases as they come, treated and untreated, usually in apparent good health, my method has given approximately 90 per cent positive *blood* reactions among men whose previous medical histories were sufficiently accurate for acceptance. Among women and children (five to eighteen years of age) the incidence has varied from approximately 50 to 80 per cent positive reactions.

In no other group of cases is a specifically sensitive method so urgently required and it is doubtful if any method can be rendered sufficiently sensitive for the detection of all cases. It is in this group that weakly positive reactions are so apt to occur and when found should not be neglected but the test repeated at least once or twice even though the physician may decide against the institution of specific medication.

Cardiovascular and central nervous system lesions are especially likely to be found and indeed, while the blood reactions may be negative the spinal fluids may show abnormal changes and especially positive complement-fixation and colloidal-gold reactions, the latter usually of the Zone II or so-called "luetic type," in anywhere from 10 to 40 per cent of cases depending largely upon the kind and amount of previous medication.

THE COMPLEMENT-FIXATION REACTION IN THE DIAGNOSIS OF TERTIARY OR CHRONIC SYPHILIS.

This great group is inseparably connected with so-called latent or concealed syphilis just discussed but I refer under the heading to those cases presenting the late, chronic or tertiary lesions detectable by combined clinical, roentgenological and laboratory examinations. The percentage of positive complement-fixation reactions for the group as a whole has been approximately 80 to 100 per cent but it is better to list them separately according to clinical classifications; as they come and go, with and without previous treatment, the *percentage of positive reactions* by my method have been approximately as follows:

	Per cent.
Chronic syphilis of the skin and mucous membranes	90 to 96
Chronic syphilis of the bones and joints	80 to 90
Chronic gastric syphilis	90 to 95
Chronic syphilis of liver and spleen	90 to 95
Chronic cardiovascular syphilis	80 to 96
Chronic syphilis of the eye, ear, nosr and throat	85 to 97
Chronic neurosyphilis (blood reactions)	70 to 100
(spinal fluid reactions)	80 to 100

Tertiary Syphilis of the Skin and Mucous Membranes.—A percentage of 90 to 96 positive reactions indicates the practical value of the method and especially the great aid it affords in the diagnosis of atrophic and pigmentary syphilids and leukodermata and their differentiation from non-syphilitic granulomas like tuberculosis, leprosy, sporotrichosis and blastomycosis as well as from such non-granulomatous lesions as psoriasis, seborrheic dermatitis, ringworm and tumors (epitheliomata, fibrosarcomata, mycosis fungoides, etc.).

The reaction has been of particular value in the diagnosis of the palmar syphilids and their differentiation from eczema, ringworm, psoriasis, etc.; also in the detection of nodular, hypertrophic and destructive syphilids of the nose, lips and mucous membrane and the differentiation of the latter from early tuberculosis, carcinoma, sclerosing glossitis, etc., in which dark-field examinations are useless.

Of course a positive reaction does not of itself prove that a lesion of the skin, lips or other mucous membrane is syphilitic. It shows that the patient *is* syphilitic and that the lesion *may* be; the microscopic examination of an excised portion may be required for final diagnosis.

Tertiary Syphilis of the Skeletal System.—The frequency of involvement of the bones and joints in acquired and congenital syphilis is receiving increasing clinical attention and the occurrence of 80 to 90 per cent positive blood reactions in late acquired and congenital infections presenting lesions indicates the great aid in diagnosis

afforded by the complement-fixation test. As stated by Stokes the provocative procedure increases the percentage of positive reactions by about 4 per cent and as shown by Chesney, Kemp and Baetjer[10] tests conducted with the *joint fluids* may be of distinct additional value since all of 10 cases, including 2 of tabes dorsalis and Charcot joints, gave positive reactions; similar results have been also reported by Jacquet and Durand[11] and others.

Chronic Syphilis of the Gastro-intestinal Tract.—In a series of 40 cases of real gastric syphilis Brown and Stokes observed 92 per cent positive blood reactions. Evidences of neurosyphilis were found in 75 per cent and they point out the special value of spinal fluid examinations in this connection. The importance of gastric syphilis to the abdominal surgeon is indicated by the 18 per cent of useless operations in their series. Stokes believes that the test possesses remarkable trustworthiness in this type of syphilis and states that false positive reactions have been so unusual in his experience as to be of negligible importance while false negatives are so rare as to render repeatedly negative reactions the strongest possible presumptive evidence against the disease.

The test has also proven very valuable in the diagnosis of syphilitic strictures of the rectum.

Syphilis of the Liver and Biliary Tract.—As stated by Stokes "the prop of clinical diagnosis in hepatic disease, so far as syphilis is concerned, is the blood Wassermann reaction, which has reduced greatly the time requirements and exactions of the former diagnosis by therapeutic test. This is indicated by the occurrence of 90 to 95 per cent positive reactions, being 90 per cent in a series studied by Stokes giving 55 per cent positive histories and lesions elsewhere in the body in 20 per cent.

"Nothing less than the application of the blood Wassermann test before operation to every patient with a surgical lesions of the abdomen would protect the patient from error in diagnosis of syphilitic liver and biliary tract—every patient who has a repeated strong positive reaction with a hepato-splenic syndrome or a gall-bladder syndrome is entitled to a therapeutic test before operation unless he is a critical surgical emergency—which should be extended to include patients with symptoms of pancreatitis and pancreatic tumor, mesenteric tumor, and so forth—while the question of a false positive tendency in carcinoma of the liver can be answered in the negative."

In this connection I may also point out the aid given in diagnosis by testing ascitic fluids since in syphilis these almost invariably yield positive reactions as reported by Roger and Sabareanu,[12] Riser,[13] Klauder and myself,[14] Belding[15] and others.

Chronic Syphilis of the Cardiovascular System.—One of the urgent needs of today in internal medicine is an improvement in our means

of diagnosis of *early* syphilis of the heart. In chronic infections my method yields from 80 to 96 per cent positive reactions, taking the cases as they come and including all types with and without previous treatment; this indicates not only the extremely valuable aid which it affords in the diagnosis of cardiovascular syphilis but the practical necessity of including the complement-fixation test in routine examinations of the cardiovascular system. In aortic insufficiency the incidence of positive reactions is approximately 88 per cent while in aortic dilatations and aneurysms the percentage is about 96.

According to Stokes a persistent *negative* reaction rules out syphilis in about 80 per cent of cases, but I believe that this is nearer 85 to 90 per cent by my technique. He has observed false positive reactions in non-syphilitic bacterial endocarditis but not by my method; all cases examined by me during the past six years have yielded negative reactions excepting one presenting syphilitic valvulitis with a superimposed streptococcus viridans infection.

Stokes has also observed an increased percentage of positive reactions following the institution of treatment (provocative procedure) and I have had a similar experience; mere repetition of the tests in cases giving primary negative reactions also increases the percentage of true positive reactions. The majority of cardiovascular cases also show abnormal spinal fluids.

Chronic Syphilis of the Eye, Ear, Nose, and Throat.—The complement-fixation test has proven an exceptionally valuable aid in the diagnosis of syphilis of eye, ear, nose and throat. Practically all cases of secondary syphilis showing iritis and early neuroretinitis give positive reactions. In late acquired syphilis with abnormal pupillary reactions, muscular paralyses or fundus changes, diplopia and failing vision due to primary or secondary changes in the optic nerves, the incidence of positive reactions is approximately 90 per cent. In primary optic atrophy of tabes dorsalis the blood reactions are approximately 85 per cent positive but the spinal fluid yields about 95 per cent reactions; in paresis the incidence on blood and spinal fluid has been 97 per cent except in well advanced paresis when positive reactions have occurred in practically 100 per cent of cases.

In congenital syphilis presenting interstitial keratitis, iritis, choroiditis, uveitis, retinitis pigmentosa, ptosis, etc., the incidence of positive reactions has been approximately 90 to 95 per cent among children under ten years of age; among older children the percentage of positive reactions has been approximately 85.

Two cases of chancre within the *nose* (1 occurring in a physician) and tested twelve and fifteen days after onset have yielded positive reactions. In the nodular and hypertrophic syphilids of the nose the reaction is positive in practically 100 per cent of cases. In late

acquired syphilis presenting gummata and osteochondritis of the nose and palate, with or without perforation, positive reactions have occurred in approximately 98 per cent of cases. In early congenital syphilis with snuffles, etc., the reaction has been positive in practically all cases, while in late congenital syphilis with osteochondritis (saddle nose) the incidence has been approximately 94 per cent among children under fifteen years of age and between 88 and 94 per cent among older children and adults.

Syphilitic involvement of the *ear* is usually an involvement of the eighth nerve and thereby a form of neurosyphilis, acute syphilitic neuritis and labyrinthitis being rare. But as stated by Stokes the development of deafness or dizziness from vestibular disturbances in young persons should be signals for blood tests.

Neurosyphilis; Variations in Blood and Spinal Fluid Complement-fixation Reactions.—In untreated acute early syphilis (late primary and secondary stages) when the blood reactions are approximately 98 to 100 per cent positive the spinal fluid reactions are positive by my method in approximately 30 to 40 per cent of cases; after treatment the incidence of positive spinal fluid reactions is reduced to about 20 per cent.

The incidence of positive reactions in late neurosyphilis varies considerably according to the degree of infection, the highest percentage occurring in the parenchymatous and the lowest in the meningo-vascular infections:

TABLE 80

	Positive blood, per cent.	Positive spinal fluid, per cent.
Asymptomatic (early and untreated)	85 to 100	30 to 40
Asymptomatic (late and untreated)	70 to 90	80 to 90
Tabes dorsalis	70 to 85	96
Paresis	98 to 100	100
Cerebrospinal syphilis	80 to 90	95 to 100

In paresis the blood and spinal fluid reactions are positive in practically 100 per cent of cases but in all other forms of late neurosyphilis the blood reaction *may* be negative when the spinal fluid reaction is positive. This is probably due to the fact that relatively larger amounts of fluid are used in conducting the tests, which thereby increases sensitiveness; also in part to the probable production of complement-fixing antibody in the tissues of the central nervous system and its higher concentration in the cerebrospinal fluid. At any rate *negative blood reactions and even repeatedly negative reactions alone never rule out the possibility of a neurosyphilitic infection* since they may occur in anywhere from 10 to 30 per cent of cases showing positive spinal fluid reactions.

The reverse of this occurs much less frequently. In practically all cases of tabes, paresis and cerebrospinal syphilis showing positive

blood reactions the spinal fluids are also positive; therefore in cases of these kinds with definite clinical signs and positive blood reactions spinal fluid examinations are not required for diagnosis although always preferred and especially since mental symptoms associated with positive blood reactions do not always indicate paresis. Moreover in some cases of vascular neurosyphilis the spinal fluid reactions are negative when blood reactions are positive, which influences prognosis and treatment.

The complement-fixation reaction has proven therefore of great value in the diagnosis of neurosyphilis and especially when applied to spinal fluids along with total cell counts, protein estimations and colloidal-gold reactions (the colloidal-mastic reaction being much less valuable in my experience). The spinal fluid examinations are also of great value in estimating prognosis and in making out therapeutic programs; a strongly positive complement-fixation reaction like 4443 associated with a paretic colloidal-gold curve like 555432000 is particularly grave and demands especially vigorous treatment even though but few or no symptoms are present.

THE COMPLEMENT-FIXATION REACTION IN THE DIAGNOSIS OF CONJUGAL AND CONGENITAL SYPHILIS.

The complement-fixation reaction has proven of great value in relation to the diagnosis of syphilis involving marriage and congenital infections of children. The virulence of the disease is apparently reduced to some extent by pregnancy and may easily escape clinical detection. Competent surveys of dispensary and hospital cases have shown positive reactions in from 2 to 8 per cent of pregnant white and in 15 to 20 per cent of pregnant colored women; in private practice the incidence is anywhere from 1 to 5 per cent with a general average of 3 to 4 per cent. Among 7768 pregnant women of Chicago including Mexicans and Negroes, Stillians observed that 7.5 per cent gave positive reactions; among 814 Negro women alone the incidence was 19.2 per cent positive reactions.

It is safe to say that not more than 20 to 25 per cent of these syphilitic individuals showed clinical evidences of the disease and yet its detection in pregnant women is of tremendous importance in relation to infant mortality. *Pregnancy itself does not give biologically falsely positive reactions in my method* and as stated by Stokes the test should be routine on all patients and should be repeated at least once or twice toward the middle and end of pregnancy. *The danger is not from falsely positive but from falsely negative reactions* and negative complement-fixation and precipitation reactions alone are never absolutely trustworthy in ruling out syphilis in pregnant women or those who have borne children. *A single negative reaction is especially untrustworthy* and syphilitic

women *after* delivery may give negative reactions. Stillians writes me that of 52 pregnant women giving positive reactions during pregnancy, 41 continued to do so after delivery; 11 gave negative reactions after delivery but all were regarded as syphilitic on the basis of other evidence.

Spinal fluid examinations are not required during pregnancy but should be done after delivery and especially if the husband has an obvious neurosyphilis since Moore and Kemp have found that 53 per cent of the wives of such men show abnormal spinal fluid changes. The relation of syphilis to miscarriage is well known and especially premature births of macerated fetuses alternating with still-born or living syphilitic children. *The complement-fixation test therefore plays a very important role in the diagnosis of syphilis and especially of latent (concealed) syphilis of wives of syphilitic men and of the husbands of syphilitic women.*

It has also shown that the apparently healthy mother of a syphilitic child is not always free or immune to the disease as originally surmised according to the *law of Colles* but that she may be actually syphilitic; likewise that the apparently healthy child of a syphilitic mother is not always free or immune according to the *law of Profeta* but actually syphilitic in the majority (but not all) of instances. In other words it would appear that some children of syphilitic mothers are free of the disease, not because they inherited an immunity, but because they escaped intra-uterine infection.

Approximately 80 per cent of the fathers of syphilitic children give positive reactions by my method and from 60 to 90 per cent of the mothers so that conjugal syphilis is demonstrable by the method in from 60 to 90 per cent of the parents of syphilitic offspring.

The method is also of inestimable value in the detection of latent or concealed syphilis of men and women prior to marriage and should always be a part of the examination required or requested before this state is entered into in the case of those known or suspected of being syphilitic and especially of known cases under or following treatment.

It has been estimated that while less than 1 per cent of the children of well-to-do parents show positive reactions that as many as 2 per cent of white and 15 per cent of colored children of the dispensary class give positive reactions with a general average of 3 to 5 per cent for our city populations. Fully 60 to 80 per cent of children of Wassermann positive mothers give positive reactions by my method when tested ten days or more after birth.

In children born with the obvious acute skin and mucous membrane lesions of syphilis the reaction is positive in practically 100 per cent of cases. Among children born of syphilitic mothers without manifest lesions and tested a *month or two later* positive reactions are observed in approximately 70 to 80 per cent; a small proportion

of these react negatively when tested immediately or within the first week after birth. The child of a Wassermann-positive mother may react positively during the first week or two after birth and then react negatively. Some physicians are of the opinion that the temporarily positive reaction is due to the transfer of antibody from the mother to the child without the latter being syphilitic, but I believe that this is a very risky assumption and prefer to regard the children as potentially syphilitic.

The incidence of positive reactions among adolescents and young adults with latent (concealed) congenital syphilis is about 40 per cent; among those with saddle noses and other skeletal lesions the incidence is about 70 per cent. Spinal fluid changes have been found in 20 to 40 per cent but the serological evidences of late and concealed congenital syphilis are so frequently negative that very thorough clinical and roentgenological examinations are usually required for diagnosis.

Therefore *it is advisable to make at least two routine blood complement-fixation reactions on all children of syphilitic parentage* namely, (*a*) the first within a few days to a week after birth and (*b*) a second about one to two months later. The first may be negative and the second positive; or the first may be positive and the second negative. In either case I regard the child as potentially syphilitic and this is a certainty if both are positive.

Cord Blood Complement-fixation Tests.—The sera of cord bloods are apt to be anticomplementary and require careful testing to avoid non-specific reactions. *A negative reaction is inadequate evidence of the absence of syphilis.* A positive reaction in my opinion means syphilis of both mother and child although some physicians are of the opinion that it indicates syphilis of the mother rather than of the child. As shown by Jeans and Cooke it is more reliable than a histological examination of the placenta since it corresponded with clinical expectation in 63.3 per cent as compared with 27 per cent of placental examinations. I thoroughly agree with Stokes that "at best the Wassermann test on cord blood is only part of the supervision with respect to syphilis which an obstetric service or hospital will provide for the protection of its clientele;" in my opinion the same is also applicable to private practice.

THE COMPLEMENT-FIXATION REACTION AS A GUIDE IN THE TREATMENT OF SYPHILIS AND AS A CRITERION OF CURE.

I have elsewhere[16] discussed these subjects in detail in relation to my test. If complement-fixation and precipitation are due to antibodies or reactionary substances produced by *Spirochæta pallida* and therefore reactions of infection as I believe the evidence indi-

cates, I see no escape from the conclusion that repeatedly positive reactions after rest periods following treatment are indices of continued infection even though they occur in individuals in apparent good health. This does not always mean that treatment should be continued merely to remove this positive quality of the serum if there are contraindications but in my opinion it *does* mean that the patient *is* syphilitic and must be carefully watched for relapses. In a fairly extensive clinical experience in the treatment of syphilis I have seen so many relapses follow cessation of specific medication among those tired of the "tyranny" of persistently positive reactions and especially weakly positives, that I believe a very heavy responsibility rests upon any physician, regardless of his experience, who deliberately ignores them in the treatment of the disease.

As expected the influence of treatment upon acute early syphilis is much more marked than in the case of late syphilis. In the great majority of cases the complement-fixation reaction is positive before treatment is begun and one of the most valuable criteria of "cure" is a persistently negative blood reaction over a period of at least two years following cessation of treatment including at least one negative spinal fluid reaction. In *primary and secondary syphilis* these results are obtained in the majority of cases although according to Moore and Kemp a too rapid reduction to negativity or a marked delay in becoming positive in the secondary stage indicates a relatively low resistance to the disease. All experienced syphilologists appear to be in agreement upon the necessity of a *minimum* amount of treatment for early syphilis regardless of the results of complement-fixation and precipitation reactions although there is considerable difference of opinion on what this should be. In *late syphilis* the strength of the reactions fluctuates greatly during treatment with a tendency to negativity; in the majority this result is obtained while in some it is not, constituting the group of Wassermann-fast cases discussed below.

Positive reactions are but one of the signs of the disease but I do not agree with Stokes that reversal to negative under treatment deserves no more weight than is accorded the healing of a visible lesion; in my experience the healing of visible lesions is a much simpler task and requires much less treatment than eradication of the whole disease to the point of persistently negative serum reactions.

The technique employed has a tremendous influence upon results. My test continues positive much longer than the original Wassermann reaction or my former method. The technique should be as sensitive as possible with specificity in order to avoid the regrettable error of undertreating the disease and it should be a quantitative reaction. In some cases the Kahn test continues to yield weakly positive reactions after my reaction becomes negative. Whether

this indicates the advisability of more treatment I am unable to state at present although the occurrence of falsely positive Kahn reactions of similar degree has lead me to ignore them when my test yields repeatedly negative reactions.

In my experience the iodides alone have had little or no influence upon reactions. Mercury by ingestion has likewise had but little effect although mercury by inunction and injection has exerted more influence. Bismuth has had somewhat more effect than mercury but much less than arsphenamin, silver arsphenamin and neoarsphenamin. In other words the influence of these compounds has been roughly in ratio to their spirocheticidal activities.

When used as a criterion of cure it is absolutely essential that persistently negative reactions over at least two years shall be insisted upon. After the last course of treatment, when it is believed that permanent negative reactions have been obtained, the tests should be done every two months during the following year, including a spinal fluid examination and every four months during the second year. In cases of chronic or late syphilis the tests should be conducted thereafter at least twice a year for several more years.

Provocative Complement-fixation Reactions.—It is commonly observed that complement-fixation reactions in late syphilis become temporarily stronger after the institution of treatment and especially with arsphenamin and its congeners. Clinical "flare up" may also occur constituting Jarisch-Herxheimer reactions and it is reasonable to assume that both are due to a stimulation of spirochetic activity.

Some have claimed that the administration of arsphenamin or neoarsphenamin to cases of late syphilis and especially latent (concealed) cases with negative blood and spinal fluid reactions provokes positive reactions of diagnostic value and a further test of cure. But there is considerable difference of opinion on both subjects. In my experience the fluctuations are sometimes to be ascribed to the influence of technique and variations in antibody and this is especially true if a *single* preliminary negative is followed by a positive reaction.

Greenbaum and Wright[17] who have reviewed the literature and studied this subject with much care have reached the following conclusions:

1. The adoption of Kolmer's standardized complement-fixation test for syphilis removes an important criticism against the use of the provocative test.

2. The specificity and increased sensitivity of the Kolmer test adds nothing to the value of the provocative test *per se*. It is possible that the effect of the increased sensitivity of this method as compared with the older tests may be, in part, counterbalanced by its increased specificity; the natural influence of the former would be to yield a larger number of positives, but the higher degree of specificity would exclude false positives.

3. When the Kolmer test is negative on first examination of children born of syphilitic parents, provocatory tests rarely aids in determining the presence or absence of syphilis.

4. The provocative test is of value in a proportion of cases as a support to clinical diagnosis, and although it offers little help when the diagnosis of a suspicious syphilitic infection is sought, yet the occasional aid it may give makes its performance imperative in some instances.

5. The provocative test is of greatest value as an added criterion concerning the persistence of the disease.

6. It should be emphasized that a negative provocative test by no means excludes the presence of syphilis. This is in accord with the interpretation of all biological tests.

In some cases presenting no clinical evidences of syphilis the injection of one dose of arsphenamin or neoarsphenamin has yielded me no conclusive evidence of provocatory stimulation and has not proven of more value than repeated blood tests without injections; the administration of three or more injections, however, has sometimes yielded undoubted provocatory reactions and especially in cases presenting some clinical evidences of syphilis. Furthermore the injection of one dose has had no value in my experience in provoking reactions in the determination of "cure" and for both purposes to be applied in exceptional cases I have employed the following method:

(*a*) The intravenous injection of 0.6 gm. neoarsphenamin or 0.4 gm. arsphenamin.

(*b*) Blood withdrawn forty-eight hours later and the test conducted; if negative.

(*c*) A second intravenous injection five days after the first.

(*d*) Blood withdrawn forty-eight hours later and the test conducted.

(*e*) If positive the result is checked by a third test. If negative, a third injection is given followed by a blood test forty-eight hours later. If negative a final test is taken five days later.

The Clinical Significance of Fixed or Wassermann-fast Reactions.—As stated by Wile and Hasely the increasing sensitivity of the complement-fixation reaction is making it increasingly difficult serologically to "cure" or arrest late cases. Stokes writes that "this point has been especially brought home since the adoption of the Kolmer technique for the Wassermann reaction in the Mayo Clinic. Patients who by the older technique had achieved apparently symptomatic and serological arrest, and were to all appearances enjoying a good therapeutic result, have now been found to be Wassermann positive on the blood by the more delicate procedure, though, as a rule, partially rather than strongly positive."

The clinical significance of persistently positive reactions is indicated by the fact that of 458 syphilitic patients who had received from 12 to 29 arsphenamin injections combined with 14 to 90

inunctions of mercury over eleven months, Stokes and Busman found cardiovascular lesions in 44 per cent, neurosyphilis in 30 per cent, osseous, hepatic, splenic and gastric lesions in 51 per cent and other types in 10 to 17 per cent. Only 10 patients or about 2 per cent presented no other evidence of syphilis.

This in my opinion is the most conclusive answer to the question. I have had but few cases of Wassermann-fast syphilis in my practice in whom some evidences of infection were not found by combined clinical, roentgenological and laboratory study and I am convinced that all require continued observation and periodic courses of treatment, the methods which I employ being described elsewhere in detail.[16]

THE COMPLEMENT-FIXATION REACTION AS AN INDEX OF RELAPSING SYPHILIS.

Finally, the *complement-fixation test is frequently and usually the first sign of relapsing syphilis* following insufficient treatment or no treatment at all. It is commonly observed weeks and months before clinical signs are evident and especially in late syphilis. For this reason *periodic blood tests (twice a year) for several years are the best means available for detecting relapses* following the dismissal of our patients from treatment, although I do not by any means under-rate thorough physical examinations for relapsing chancres (monorecidives), delayed and recurring secondaries, neurorecurrences, precocious tertiarism, the development of cardiovascular, ocular, visceral and skeletal lesions, the birth of syphilitic children, etc. Stokes states that the blood Wassermann reaction alone cannot expect to detect more than 70 per cent of relapses but I am sure this figure is too low. It is probably correct for cases showing no clinical evidences but when lesions are present the incidence of positive reactions is over 90 per cent and of great confirmatory value in diagnosis.

THE COMPLEMENT-FIXATION REACTION IN RELATION TO LIFE INSURANCE.

While the average total mortality of life insurance companies runs between 60 and 85 per cent of actual to expected deaths, the mortality-rate among syphilitic individuals accepted after two years of treatment and an absence of all symptoms including negative Wassermann reactions over at least a period of one year, has been according to Dr. A. B. Hobbs of the New York Life Insurance Company, from 122 to 149 per cent taking 100 per cent as the expected average according to the American Mortality Select Table of expectancy. In 1919 the Penn Mutual Company reported 145 per cent at ages of entry under forty years and 301 per cent at forty years and over,

which shows the lack of longevity among syphilitics. The Equitable reported 160 per cent and the Mutual Life 158 per cent on life policies. As stated by Dr. Hobbs, where there is satisfactory evidence as to thorough treatment and cure and no other impairments, an average mortality rating of from 135 to 140 per cent will probably cover carefully selected cases. He would not hesitate to rate the "latent" cases of syphilis at somewhere over 150 per cent. In cases with minor but significant impairment when syphilis is considered cured, such as tuberculosis in the family history, the mortality is increased approximately to 170 to 185 per cent. In alcoholic cases, but not to excess, the mortality is increased to 225 per cent due to a very heavy death-rate from heart disease, cirrhosis of the liver and suicide. In syphilitic overweights (with an abdominal girth more than 2 inches greater than the chest at rest) a ratio of 230 per cent has been observed, cerebral hemorrhage, nephritis and heart disease being the principal causes of premature death in this class.

These figures have not been based upon the Wassermann reaction alone but indicate that actuaries should figure on at least a death-rate of 135 to 140 per cent and certainly show the importance of syphilis as a substandard risk in life insurance; likewise, as I pointed out some years ago,[18] the importance of the complement-fixation test in its detection, particularly in treated and latent or concealed cases where the test should possess the maximum of specific sensitiveness and be conducted with particular technical care since clinical detection may be very difficult and indeed impossible and the histories sometimes misleading.

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CHAPTER XLIII.

SENSITIVENESS, SPECIFICITY AND PRACTICAL VALUE OF THE KOLMER COMPLEMENT-FIXATION REACTION IN GONOCOCCUS DISEASES.

The gonococcus complement-fixation test serves a very useful purpose under well-defined conditions although its indiscriminate use has resulted in dissatisfaction.

SENSITIVENESS.

In the first place it is not as sensitive as the complement-fixation reaction in syphilis for the very simple reason that but little antibody is produced when the disease is limited to a circumscribed and superficial area in simple urethritis. Furthermore, time is required for antibody production.

For these reasons the reaction is rarely positive during the first two weeks of acute urethritis of either sex and bacteriological examination of smears and cultures (especially the former) constitutes the best means for laboratory diagnosis. The earliest positive reactions in acute gonorrheal urethritis of males (first attacks) which I have so far observed have been during the fourth week and all have had posterior urethral infections with probable early involvement of the prostate gland. I have not observed any positive reactions in the mild infections presumably limited to the anterior urethra and while I have had much less experience with acute gonorrhea of women yet the results have indicated that positive reactions are not to be expected during the first few weeks of gonococcus urethritis and its associated bartholinitis.

Likewise in acute gonorrheal ophthalmia of the new-born the reactions have been uniformly negative and I believe the general statement may be made that *the complement-fixation test is without value in the diagnosis of acute circumscribed gonococcus infections;* in these bacteriological examinations are to be preferred in much the same manner as in the diagnosis of the chancre by dark-field microscopy.

But in more wide-spread gonococcus disease the reactions are very frequently positive and of great value in diagnosis. I refer to chronic urethro-prostatitis of males with and without acute exacerbations, chronic cervicitis and salpingo-ovaritis of adults, chronic vaginitis and cervicitis of children and especially in arthritis, iritis and endocarditis. In these bacteriological diagnosis becomes increasingly difficult and very frequently impossible, especially if the suc-

cessful cultivation, isolation and identification of the gonococcus is required. Indeed the complement-fixation test may disclose the presence of foci of hidden infection in the genital adnexa, joints and iris when bacteriological diagnosis is impossible and prove a very valuable part of the survey required for ascertaining whether or not recovery has occurred and especially in relation to preparation for marriage.

The incidence of positive reactions is greatly influenced by the sensitiveness of the antigen and technique employed as is always the case when small amounts of antibody are to be detected. For this reason *the method must be as sensitive as possible with specificity* but even under the best of conditions *the tendency to falsely negative reactions is ever present and renders a negative reaction of very little value in excluding gonococcus disease* except in the case of arthritis. In other words, the highest percentage of positive reactions is observed in gonococcus arthritis which in turn renders a negative reaction of increased value in excluding this infection.

Positive Reactions in Gonococcus Infections of Men.—Insofar as my method is concerned the *percentage of positive reactions* in gonococcus disease of men is approximately as follows, based upon the accumulation of data during the past six years and including an intensive study of the subject in coöperation with my colleague Dr. Charles deT. Shivers:

	Per cent.
Acute anterior urethritis (first five weeks)	6 to 10
Subacute antero-posterior urethritis	24
Chronic posterior urethritis	35
Chronic urethritis with acute exacerbations	80
Chronic prostatitis	60
Chronic epididymitis	80 to 90

Positive Reactions in Gonococcus Infections of Women.—I have had much less experience with the method in these infections but the *percentage of positive* reactions have been approximately as follows:

	Per cent.
Acute urethritis and bartholinitis (first four weeks)	8
Subacute and chronic urethritis (one to six months)	60
Chronic pelvic infections with and without exacerbations	71

In the acute infections smears may be expected to disclose the presence of the gonococcus in about 60 per cent of cases but in the chronic infections but 15 to 40 per cent show the organism in smears and cultures; *the complement-fixation test is therefore superior to bacteriological examination in the diagnosis of subacute and chronic gonorrhea of women and has proven especially valuable in institutional work.* Thinking that it may be possible to demonstrate gonococci in vaginal exudates by using the pus as antigen against horse anti-gonococcus sera I suggested a study of this kind to Dr. R. S. Patterson but his results were almost entirely negative[1] so that a reversed complement-fixation test is without diagnostic value.

Positive Reactions in Gonococcus Vaginitis of Children.—In acute vaginitis as encountered in hospital outbreaks the reactions are almost always negative but in chronic infections extending over months and years the percentage of positive reactions has reached as high as 60 per cent in some groups and indicating the probability of associated cervicitis and even higher infections. In acute infections diagnosis is best made by bacteriological methods but in chronic infections the complement-fixation test is sometimes of value and especially in those of persistent discharge with negative bacteriological findings; a positive reaction establishes the presence of the disease although negative reactions do not exclude it.

Positive Reactions in Gonococcus Arthritis, Iritis and Endocarditis.—It is commonly stated that in undoubted gonococcus *arthritis* the reaction is positive in about 100 per cent of cases. During the past six years I have not been able to test any falling into this category but of 9 cases in men very probably gonococcic and associated with chronic urethro-prostatitis, the reactions were positive in 8 or approximately 90 per cent. Furthermore the reactions were strongly positive in 6 of these and strongly positive reactions are the exception rather than the rule in gonococcus complement-fixation tests. *A positive reaction, however, does not always establish the diagnosis of gonococcus arthritis if there are foci of infection elsewhere as in the genital tract, although a strongly positive reaction is pretty strong evidence; a negative reaction, however, is weighty evidence against the gonococcic origin of an arthritis in men.*

A similar situation pertains to *iritis*. I have never seen undoubted cases in women but among men with chronic urethral infections the incidence of positive reactions has been approximately 80 per cent (5 positive reactions in 7 cases) so that the complement-fixation test is probably of more value in the differential diagnosis of chronic recurrent iritis than generally surmised. Positive reactions were also observed in 3 cases of *endocarditis* complicating urethral infections of young men.

SPECIFICITY.

The gonococcus complement-fixation reaction is biologically specific. If due care exercised against anticomplementary reactions and the use of a too closely adjusted hemolytic system, *positive reactions including the weakly positive ones are invariably true and specific.* However this does not exclude the possibility of falsely positive reactions due to errors in technique although the whole tendency is much greater in the direction of falsely negative than toward falsely positive reactions.

In infections of the urethra and adnexa of both sexes associated with a secondary infections showing the presence of other Gram-negative diplococci, staphylococci, colon bacilli and other organ-

isms the reactions when conducted with *pure antigens of gonococci* are biologically specific. Even the presence of large amounts of syphilis antibody giving strongly positive Wassermann reactions does not give falsely positive gonococcus reactions with the antigens in common use which is an extremely important practical point in relation to serum diagnosis.

PRACTICAL VALUE.

Despite the fact that the complement-fixation test is of little or no value in the diagnosis of acute gonococcus infections of either sex, and despite the fact that a negative reaction does not always exclude the disease in chronic infections, nevertheless the test possesses considerable practical value when properly conducted in the following conditions:

1. In the diagnosis of the acute complications of gonorrhea and of the acute exacerbations of chronic urethro-prostatitis since positive reactions occur in from 80 to 90 per cent of cases and bacteriological diagnosis is extremely difficult and sometimes impossible.

2. In the detection of latent foci of gonococcus infection in the epididymis, prostate, etc., of men in the absence of urethral discharge. In this connection it is always worthy of being included in *examinations for marriage* when the individual is known to have had gonorrhea even though no discharge had been observed for weeks and months. A positive reaction is acceptable as evidence of residual infection which may be rendered active and infectious by many different factors.

3. As one test for the cure of a gonococcus infection and especially of those *previously yielding positive reactions*. Just how much time is required for the disappearance of antibody after the eradication of infection cannot be stated and doubtless varies a great deal, but tests yielding persistently positive reactions at intervals of two months are almost surely the result of residual infection and from 20 to 25 per cent of individuals clinically well and free of discharge may continue to yield positive reactions for varying periods of time and some indefinitely. In this connection it must be remembered that the administration of gonococcus vaccine and antigonococcus serum may be responsible for positive reactions but these are ordinarily eliminated within six to eight weeks after the last dose.

4. In the diagnosis of gonococcus pelvic disease of women and its differential diagnosis from inflammatory disease of other origin, tumors, etc.

5. In the diagnosis of gonococcus arthritis, iritis and endocarditis in which the test is particularly valuable.

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CHAPTER XLIV.

SENSITIVENESS, SPECIFICITY AND PRACTICAL VALUE OF THE KOLMER COMPLEMENT-FIXATION REACTION IN TUBERCULOSIS.

As stated in Chapter I, tuberculosis was the first disease in which the Bordet-Gengou phenomenon of complement-fixation was tried by Wassermann and his colleagues about twenty-three years ago. Since then a very large literature has accumulated which up to 1915 has been excellently reviewed by Stimson[1] and from then up to 1925 by Wadsworth, Maltaner and Maltaner.[2] The reported results have varied greatly according to the numerous antigens and methods employed as, likewise, opinions concerning the value of the test; certainly the very enthusiastic reports of about ten years ago have not been confirmed and the test has not proven by any means as valuable in the diagnosis of tuberculosis as has the Wassermann reaction in syphilis.

SENSITIVENESS.

Humoral antibodies are not produced in high degree in tuberculosis; the immunological changes are predominantly cellular and for this reason large amounts of complement-fixing antibody are not ordinarily found in the blood. I believe that this constitutes the chief reason for the lack of better results with the complement-fixation, precipitation and agglutination reactions in all forms of tuberculosis and *if the method is one combining only that degree of sensitiveness consistent with specificity, positive reactions have a much greater value in establishing the diagnosis of tuberculosis than do negative reactions in excluding the infection; in other words, the reaction has a greater positive than negative value when syphilis can be excluded.* Furthermore the preparation of antigen is still a debatable subject although it would appear that the lipoids of the bacilli and especially those insoluble in acetone are of importance as discussed in Chapter XV. In 1924 Wassermann[3] described a new antigen prepared by extracting tubercle bacilli with tetraline (a tetrahydrated naphthaline) in a shaking machine for several weeks to the point where they are no longer acid-fast and then adding a small amount of lecithin to the extracted bacilli. Pinner[4] has recently reviewed the literature and in a study of 646 patients found 71.5 per cent positive reactions in active tuberculosis. Positive reactions

were also observed in clinically arrested cases and in a number of clinically non-tuberculous patients, especially women before and after confinement. Indeed this and other studies have not by any means confirmed Wassermann's report or materially improved the status of the reaction.

In 1923, with the coöperation of Dr. Andrew Callahan and the assistance of Miss Elizabeth Yagle, I applied the test described in Chapter XXXII to the study of 112 individuals in the tuberculosis wards of the Philadelphia General Hospital. Six sera were anti-complementary and therefore excluded; the results with the remaining 106 were as follows:

Incipient pulmonary tuberculosis (12)	76 per cent positive
Moderately and far advanced (74)	81 per cent positive
Surgical tuberculosis (bones and joints (16)	67 per cent positive
Chronic pulmonary with laryngeal ulcers (3)	66 per cent positive
Tuberculous meningitis (1)	Positive
Clinically non-tuberculosis and non-syphilitic controls (171)	None positive
Clinically non-tuberculosis but syphilitic controls (30)	10 per cent positive

The incipient cases showed slight initial lesions of the apices of the lungs with no complications, slight or absent expectoration and with or without bacilli in the sputum. The clinically non-tuberculous and non-syphilitic controls were selected largely from my laboratory staff and students. The clinically non-tuberculous but syphilitic controls were selected from the clinic of Dr. Jay F. Schamberg and with few exceptions were cases under treatment; all gave moderately to strongly positive Wassermann reactions.

The 67 to 81 per cent positive reactions compares quite favorably with the incidence of positive reactions reported during the past few years with other methods although not as good as reported in 1914 to 1917 by various workers who evidently did not use their methods with a sufficiently large or varied group of patients to bring out the negative results and limitations.

Kilduffe[5] in a study with Dr. Shilene of 104 cases of pulmonary tuberculosis by my method and antigen has reported essentially similar results as follows:

"Of the 78 cases with positive sputum, 57 or approximately 61 per cent gave positive fixation reactions of varying degrees. The total number of positive reactions in the entire series was 67 or 64 per cent; 36 or 34 per cent were negative on a single examination.

The results in general may be thus tabulated:

Sputum positive and complement-fixation positive	57 or 54.0 per cent
Sputum negative and complement-fixation positive	22 or 21.0 per cent
Sputum positive and complement-fixation test negative	14 or 1.3 per cent
Sputum negative and complement-fixation test negative	10 or 0.09 per cent
Anticomplementary	1 per cent
Total positive complement-fixation reactions	67 or 64.0 per cent
Total negative complement-fixation reactions	36 or 34.0 per cent

These results, it must be remembered, represent the results of a single examination of the serum in each instance.

The reactions varied in strength from very strongly positive by the Kolmer scale (fixation in 4 or all 5 tubes of the series), to weakly positive (fixation in the first tube only). They were distributed as follows:

Moderately advanced cases.		Far advanced cases.	
Very strongly positive	11	Very strongly positive	10
Strongly positive	16	Strongly positive	6
Moderately positive	11	Moderately positive	7
Weakly positive	3	Weakly positive	2

While there was a distinct relation between the degree of activity and the strength of the reaction this, as might have been expected, was markedly modified by the ability of the patient to react.

The cases in which both the sputum examination and the complement-fixation test were negative are of some interest. Two were diagnosed as asthma and noted as without the physical signs of tuberculosis; 1 was a chronic empyema; 1 a case of tuberculosis of the spine, far advanced; 1 practically moribund; 1 a case of tuberculous enteritis; 1 an "arrested incipient;" 1 incipient; 3 chronic fibroid cases; and the remainder moderately advanced cases of pulmonary tuberculosis in fair clinical condition.

Of the 14 cases with a positive sputum and a negative fixation reaction, 6 were far advanced in type and the remainder moderately advanced cases."

Woolley and Petrik[6] employing a modification of my method using 10 instead of 2 units of antisheep hemolysin and a Petroff glycerin extract antigen have reported the following results in a study of 200 cases:

Classification.	Number.	Positive fixation, per cent.	Positive sputum, per cent.
Minimal	13	61	36
Moderately advanced	57	81	72
Far advanced	110	88	89
Non-clinical tuberculosis	10	40	0
Non-tuberculous	10	40	0

Six of the 8 positive reactions among the 20 clinical non-tuberculous cases were + or ++ reactions. Cross fixation with syphilitic sera were rarely observed. The percentage of positive reactions with clinically tuberculous patients was distinctly higher than with my antigen but this advantage was offset by the occurrence of so many positive reactions among clinically non-tuberculous individuals.

Ogawa[7] in an exceptionally interesting and valuable study of the complement-fixation and precipitation reactions conducted with tuberculous pleural exudates observed the following results with my method employing exudates (Chapter XXXVIII) and a Petroff antigen:[8]

"1. Of a series of 12 tuberculous pleural exudates, 42 per cent yielded weakly positive precipitin and 92 per cent well-defined positive complement-fixation reactions.

"2. Human pleural exudates and transudates of non-tuberculous origin yielded uniformly negative precipitin and tuberculosis complement-fixation

reactions. In syphilis, however, positive reactions may occur due to the presence of the "reagin" in the exudate unless precautions are taken to remove the lipoids from the antigen of tubercle bacilli.

"3. With the pleural exudates secured fifteen days or longer after experimental tuberculous pleuritis in guinea-pigs, 8 per cent yielded weakly positive precipitin reactions and 89 per cent strongly positive complement-fixation reactions.

"4. In experimental tuberculous pleuritis and pericarditis of guinea-pigs and rabbits, precipitins and complement-fixing antibodies are not usually found earlier than twelve days after infection.

"5. These results indicate that in the exudates of tuberculous pleuritis, precipitins and especially complement-fixing antibodies are found in a large percentage and that a sensitive complement-fixation test with special attention to certain technical features, may prove a valuable practical aid to diagnosis."

In a subsequent study of my method and antigen in 263 cases of pulmonary tuberculosis of varying duration and severity Ogawa[9] observed approximately 79 per cent positive reactions, the majority occurring in advanced infections; of 50 pleural fluids positive reactions were observed with 38 or 76 per cent. Positive reactions also occurred with the sera of syphilitic individuals and likewise with the sera of some clinically non-tuberculous individuals. The latter may have been due to the acquisition of anticomplementary properties on the part of the antigens during transit from Philadelphia to Berlin and Mukden where the studies were made.

SPECIFICITY.

To the best of my knowledge all tuberculosis antigens are capable of yielding non-specific or cross complement-fixation reactions with a small percentage of the sera of clinically non-tuberculous syphilitic individuals. Certainly this has been my experience in a study of antigens prepared by twenty or more different methods. These reactions are due to the fact that the lipodophilic syphilitic antibody is capable of fixing complement with the lipoids of tubercle bacilli in much the same fashion as with tissue lipoids. Therefore I believe it is advisable to remove some of these lipoids in preparing antigen of tubercle bacilli although this admittedly reduces the sensitiveness of the extracts to some extent, because some of these lipoids are apparently likewise concerned in the tuberculosis complement-fixation reaction; but even antigens prepared of tubercle bacilli deprived of acid-fastness by different lipoid solvents are capable of yielding these non-specific reactions with sera containing large amounts of syphilis antibody.

With my antigen these reactions occur with approximately 10 per cent of sera of syphilitic individuals as reported by Kilduffe[10] and observed likewise in my own work. They occur almost always with sera yielding strongly positive Wassermann reactions; sera

yielding weakly positive reactions almost always react negatively with the tuberculosis antigen unless the individual is likewise tuberculous. *But their occurrence renders it necessary in my opinion to conduct the Wassermann reaction routinely with all sera or pleural exudates submitted for the tuberculosis complement-fixation test; when the former yields a positive reaction great caution is required in the interpretation of a positive tuberculosis reaction.*

Otherwise the tuberculosis complement-fixation reaction possesses an extremely high degree of specificity. It is true, of course, that unexpectedly positive reactions occur with the sera of 3 to 10 per cent of apparently healthy individuals, but if technical errors can be excluded the burden of proof rests upon the clinician to prove that incipient tuberculosis is absent. *In the absence of syphilis and technical error the tuberculosis reaction is far more likely to err on the side of falsely negative reactions than on the side of falsely positive reactions.* Possibly leprosy may give falsely positive reactions because it is likewise caused by an acid-fast bacillus but I have had no experience with it in this relation and furthermore the disease should present no great difficulties in clinical diagnosis.

Of greater importance is the question of whether or not tuberculosis is capable of giving falsely positive Wassermann reactions. With fully cholesterolized antigens and the sera of active febrile cases of pulmonary tuberculosis, I believe that such reactions may occur but they have not occurred in my experience with my new antigen and syphilis complement-fixation method.

PRACTICAL VALUE.

As previously stated the tuberculosis complement-fixation test has not fulfilled the extravagant claims and predictions voiced some ten years ago but nevertheless is not without well defined practical value under certain conditions. *Single negative reactions are of almost no value in excluding tuberculosis* just as single negative Wassermann reactions are of much less value than several negatives in excluding syphilis. These falsely negative reactions are probably due in large part to a poverty of humoral antibodies in tuberculosis but likewise in part to lack of antigenic sensitiveness on the part of extracts and to variation in the proper quantitative relationship among antigen patient's serum and complement for the maximum of fixation. Nevertheless *positive reactions are of distinct diagnostic aid* when technical errors can be eliminated:

1. This is especially true in the diagnosis of early or *incipient tuberculosis of the lungs* among non-syphilitic individuals yielding negative Wassermann reactions. About 60 per cent yield positive reactions and a positive reaction is apt to be of distinct aid in the diagnosis of cases presenting indefinite clinical and roentgenological

evidences of infection although *a negative reaction is absolutely worthless in excluding the disease.* The value of this aid in diagnosis is in direct ratio to clinical skill in the detection of incipient tuberculosis; obviously it may be of little aid to the expert but apt to be of much more service to the general practitioner. Just how soon positive reactions may be observed in human beings after infection is impossible to state; the lower animals have reacted in from ten to twenty and as early as four days after experimental infection.

2. In *moderate or advanced tuberculosis of the lungs* positive reactions may be expected in from 60 to 90 per cent of cases. When syphilis can be excluded a positive reaction is confirmatory evidence of the presence of the disease, but obviously the test is not usually required in diagnosis except in a small percentage of clinically doubtful cases where actinomycosis, non-tuberculous pulmonary abscesses from foreign bodies or infection, malignancy, etc., require consideration. Here again however, a negative reaction does not exclude tuberculosis although a positive is very strong evidence of its presence.

3. The test is also of undoubted value in the diagnosis of *tuberculosis of bones and joints* in non-syphilitic individuals although the usual coincidence of pulmonary lesions frequently involves the question of whether or not they are responsible for the reactions. This is especially true in *tuberculous laryngeal ulcers.*

4. In *tuberculous nephritis* positive reactions are of aid in diagnosis when pulmonary lesions are inconspicuous or clinically absent and syphilis can be excluded; when moderate or advanced pulmonary lesions are present it is natural to ascribe positive reactions to them.

5. That the test may be of value in aiding the diagnosis of *tuberculous meningitis* is indicated by the positive reaction reported by Kilduffe[11] employing spinal fluid.

6. The test is of decided value in the diagnosis of *tuberculous pleurisy* when the exudate as well as the blood serum is tested and negative Wassermann reactions with both are observed.

7. Some observers have stated that the strength or degree of complement-fixation is acceptable as a measure of the activity of tuberculous lesions. Others have denied this interpretation. Obviously the degree of fixation is subject to so much variation according to the antigenic sensitiveness of the extract, the fixability of the complement, kind and duration of primary incubation and other technical factors that a lack of uniformity of opinion is to be expected. For my own part I regard the strength or degree of complement-fixation in tuberculosis as a measure of the degree of infection rather than of immunity as likewise in the case of the syphilis reaction. If this is true the degree of complement-fixation in tuberculosis is a gauge of infection under the limitations imposed by technique although the reactions may not parallel clinical conditions

just as the strength of the Wassermann reaction frequently fails to run parallel with the clinical status of some cases of syphilis. Therefore I regard a persistently strongly positive tuberculosis reaction as indicative of continued bacillary activity.

8. Just how much time is required for the disappearance of antibody following the clinical "cure" of tuberculosis cannot be stated in accurate terms but may be as long as one or two years.

9. Latent tuberculous lesions presenting negative complement-fixation reactions may be reactivated and made to yield positive reactions by various provocatory agents including the injection of tuberculin and vaccines and by excessive exercise.

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CHAPTER XLV.

SENSITIVENESS, SPECIFICITY AND PRACTICAL VALUE OF THE KOLMER COMPLEMENT-FIXATION REACTION IN TYPHOID FEVER AND OTHER BACTERIAL DISEASES.

COMPLEMENT-FIXATION IN TYPHOID FEVER.

Curiously the complement-fixation reaction has never received the attention that it deserves in the serum diagnosis of typhoid fever. This is probably due to the early popularity of the agglutination test and its greater simplicity, especially the microscopic test with dried blood submitted on paper, but the complement-fixation reaction is actually more specific, more sensitive and probably of greater value in the diagnosis of typhoid fever in individuals previously vaccinated.

First used by Bordet and Gengou[1] as early as 1901, quite a literature has accumulated which has been summarized by Garbat,[2] who has likewise studied the reaction very extensively in relation to the diagnosis of the disease and of the carrier state.

Garbat has emphasized the importance of using a *polyvalent antigen* which is corroborated by Hooker's work showing the probable existence of serological strains of the typhoid bacillus. I have generally used an antigen prepared of the Rawling's strain and that which has been in use in the Department of Health Laboratories of Philadelphia for Widal tests for over twenty-five years.

Specificity.—While the sera of normal unvaccinated individuals may give positive Widal reactions due to the presence of normal agglutinins, they do not yield positive complement-fixation reactions in the amounts employed in my test.

Furthermore, other diseases do not appear to interfere with specificity. Doubtless the serum in paratyphoid fever may give positive reactions with typhoid antigen but tuberculosis, syphilis and pneumonia have not produced falsely positive reactions in my experience which is particularly important in acute miliary tuberculosis in relation to differential diagnosis. Singer[3] suggests that *Bacilli enteriditis* (Gaertner) infections may give positive reactions because of the similarity of the bacterial receptors.

Sensitiveness.—Roughly 50 per cent of individuals with typhoid fever give positive reactions between the first and second weeks of the disease and between 90 and 95 per cent after the second week.

In my experience positive reactions have been frequently observed several days to a week or more before positive Widal reactions occurred and I believe that the complement-fixation reaction is somewhat more sensitive. Positive reactions may be delayed for four to six weeks but all of 11 cases which I have been able to examine have yielded positive reactions sometime during the course of the disease. Garbat has found the reaction positive during convalescence and the only means of serum diagnosis in as high as 9 to 15 per cent of clinically doubtful cases; in 21.5 per cent the reaction gave valuable assistance in diagnosis.

Relation to Relapses and Carriers.—Garbat has expressed the opinion that the duration of positive reactions is dependent upon the duration of the initial typhoid fever. About 30 per cent react negatively by the end of the second month of normal temperature and 60 per cent at the end of six to eight months.

Eighty per cent of recurring and relapsing cases gave strongly positive reactions.

Eighty-five per cent of carriers gave positive reactions and *a persistently positive reaction beyond six months of normal temperature is strong presumptive evidence of a carrier state although negative reactions do not exclude this possibility* since he found 15 per cent carriers giving negative reactions. In my opinion positive reacting carriers are apt to show active typhoid sequelæ while negative reactors carry typhoid bacilli in the gall-bladder without inflammatory reactions.

Relation to Typhoid Vaccination.—Vaccination with the usual three doses of triple vaccine results in the production of small amounts of complement-fixing antibody in the majority of individuals.

Garbat has found that they disappear after a month or two although Berge and myself[4] have observed positive reactions persisting as long as four months and, in one instance, for about two years. Vogelin and Gerneg[5] state that they generally disappear within six months.

Since the immunity following vaccination is believed to last for two to three years it is apparent that it bears no relation to the complement-fixation reaction and the latter lends no particular aid in solving the perplexing question of when to revaccinate those anxious to prolong their immunity. In my opinion a positive reaction may be safely accepted as evidence of the presence of protective antibodies but a negative reaction does not indicate their absence.

If symptoms of typhoid fever develop in a vaccinated individual the complement-fixation test is of greater value in diagnosis than the agglutination test. Positive reactions occurring one year or more after vaccination are generally evidence of infection and espe-

cially if repeated at intervals of three to five days by a quantitative method as described in Chapter XXXII, with increasing degrees or strength of the reactions.

COMPLEMENT-FIXATION IN PARATYPHOID FEVER.

Judging from experiments with immunized rabbits the paratyphoid bacilli A and B are just as active antigenically in producing complement-fixing antibody as the typhoid bacillus, although I have had no experience with the reaction in the diagnosis of paratyphoid fevers of human beings.

By using a quantitative method rabbit paratyphoid immune sera are readily differentiated from rabbit typhoid sera and I surmise that the differentiation is likewise possible with human sera by the same method.

COMPLEMENT-FIXATION IN CHOLERA.

Amaka and Kojima[6] have reported approximately 50 per cent positive reactions in a series of 34 cases of Asiatic cholera. Of 17 carriers of the bacillus 5 or approximately 30 per cent are reported to have reacted positively. Very severe cases have generally succumbed before positive reactions could occur.

They have also observed positive reactions with the rice water stools as antigen tested with anticholera serum; Nedrigailoff[7] and other investigators have also stated that this method is sometimes superior to the usual bacteriological methods for detecting cholera bacilli in feces.

COMPLEMENT-FIXATION IN WHOOPING COUGH.

I have had no experience with the new method in the diagnosis of whooping cough and have elsewhere[8] reviewed the literature indicating that positive reactions are not usually observed until the paroxysmal stage is reached, when about 50 per cent of sera react positively (Park). For this reason the test is said to possess little or no value in early diagnosis although sometimes of aid in the diagnosis of atypical cases.

COMPLEMENT-FIXATION IN INFLUENZA.

The complement-fixation test with antigens of *Bacillus influenzæ* have indicated the primary or important secondary role of the organism in epidemic influenza as shown by the results of Rappaport,[9] Trist, Yagle and myself,[10] Dimarid,[11] Cooke,[12] Howell and Anderson[13] Wollstein,[14] Fry and Lundie.[15] Positive reactions occur early enough to render material aid in diagnosis. Approximately 20 to 50 per cent of sera yield positive reactions, especially in pneumonia cases and during convalescence; Trist, Yagle and I also observed

about 38 per cent positive reactions with antigens of hemolytic streptococci from throat cultures. Fry and Lundie have reported about 20 per cent positive reactions in controls. Cooke observed positive reactions especially among older children and adults; children from one to five years of age reacted much less frequently. Gay and Harris[16] found 40 per cent positive reactions with the sera of individuals vaccinated against *Bacillus influenza*.

COMPLEMENT-FIXATION WITH BACILLUS ABORTUS ANTIGENS.

The increasing frequency with which *Bacillus abortus* (Bang) of contagious abortion of cows has been found in cow and goat milks and in the blood and organs of infected human beings, the literature being recently reviewed by Moore and Carpenter[17] along with the report of human infections, two of whom were possibly infected by milk, has rendered the complement-fixation test for this infection of considerable interest especially in the study of undulant and atypical fevers and joint trouble of doubtful etiology. As shown by Evans[18] there may be a close relationship between *Bacillus abortus* and *Bacillus melitensis* of Malta fever.

Since *Bacillus abortus* may produce lesions in the lower animals and particularly of the osseous system resembling tuberculosis the 17 per cent. positive complement-fixation reactions among children, most of whom were diagnosed clinically as tuberculosis and rickets, reported by Larson and Sedgwick,[19] is deserving of further investigation; Williams and myself[20] observed negative reactions with the sera of 50 aborting women tested with antigens of *Bacillus abortus* (Bang) and *Bacillus abortivo-equinus*.

COMPLEMENT-FIXATION IN CHANCROID.

Gallia,[21] Ito[22] and Teissier, Reilly and Rivalier[23] have reported as high as 90 per cent and more positive reactions with antigens of *Bacillus ducrey* but Assis[24] and more recently Carvalho[25] have been unable to confirm these earlier observations. The latter employed five different antigens with negative reactions in 94.8 per cent of cases. The positive reactions observed in 5.2 per cent occurred in syphilitic individuals yielding strongly positive Wassermann reactions and were evidently due to the fixation of complement by syphilis antibody and the lipoids of the bacillary extracts.

I have often thought that the complement-fixation test may be of value in the differential diagnosis of chronic venereal suppurations even though the one to two weeks required for antibody production would render it useless for differential diagnosis between chancre and chancroid, but apparently little or no antibody is produced in chancroid while syphilis antibody may yield non-specific reactions.

COMPLEMENT-FIXATION IN STREPTOCOCCUS INFECTIONS.

Arthritis.—Hastings[26] has reported 50 to 60 per cent positive reactions in deforming arthritis and arthritis deformans, including cases of Still's disease, with antigens of *Streptococcus viridans* although he considered the reactions too weak and unsatisfactory for practical diagnostic purposes. Burbank and Hadjopoulos[27] however, employing active serum and various antigens of hemolytic and non-hemolytic streptococci (viridans type), staphylococci and other organisms isolated from the throats, sputa, feces, etc., of arthritic individuals, believe the tests of considerable diagnostic and prognostic value; also of value in vaccine treatment as indicating the kind of organisms to employ according to the positive reactions observed. Zabriskie[28] states that they are also of value in the study of the secondary infections incident to primary foci in the teeth and gums.

Endocarditis.—Major[29] has reported a positive reaction in a case of *Streptococcus viridans* endocarditis and Aschner[30] has likewise observed positive reactions in subacute infections which may be of aid in diagnosis with antigens of heart strains of streptococci.

Scarlet Fever.—While in 1912 I observed[31] but 11.2 per cent positive reactions with antigens of streptococci isolated from scarlet fever throats, Dick[32] has observed as high as 54 to 88 per cent positive reactions with different antigens of *Streptococcus hemolyticus* from scarlet fever cases.

In a more recent study by Miss Rule and myself with specimens kindly furnished by Dr. S. S. Woody, Medical Director of the Philadelphia Hospital for Contagious Diseases, the following results were observed with 21 sera from cases varying from six to fourteen days after the onset of the disease:

Wassermann reactions (new method)	Negative in all
Reactions with antigen of scarlet fever toxin	97 per cent positive
Reactions with antigen of scarlet fever streptococcus	57 per cent positive
Reactions with antigens of control streptococci	17 per cent positive

In a second series of 25 cases varying from five to forty-two days after the onset of the disease the results were as follows:

Wassermann reactions (new method)	All negative
Reactions with antigen of scarlet fever toxin	100 per cent positive
Reactions with antigen of scarlet fever streptococcus	76 per cent positive
Reactions with antigens of control streptococci	8 per cent positive

Positive reactions occurred as early as the fifth day after the appearance of rashes and the test may be of diagnostic value in addition to lending further support to the etiological relationship of the streptococcus to the disease. But we likewise observed posi-

tive reactions with as high as 30 to 40 per cent of control sera and the antigens of the scarlet fever toxin; all of these however, occurred with the sera of adults giving negative Dick skin reactions which opens up the possibility of their being due to the presence of natural scarlet fever antibody.

COMPLEMENT-FIXATION IN SMALLPOX AND VACCINIA.

I have elsewhere[33] reviewed the literature upon the complement-fixation reaction in smallpox and after vaccination and reported the following results of an investigation:

1. The sera of rabbits inoculated with cowpox virus yielded positive complement-fixation reactions with salt solution antigens of cowpox and smallpox viruses in seven to eight days after vaccination.

2. The antibody of cowpox virus in the sera of vaccinated animals showed a distinct and close biological relationship to the antigen of variola in complement-fixation experiments.

3. Of 13 individuals vaccinated with cowpox virus from seven days to ten years previously and whose sera yielded negative Wassermann reactions, positive reactions with salt solution antigens of cowpox virus were observed with 4 or 22 per cent. The serum of 1 of these 4 persons yielding positive reactions with cowpox virus reacted positively with a salt solution antigen of variolous material. The sera of unvaccinated persons reacted negatively with all antigens.

4. Of 17 persons suffering with mild smallpox, the sera of 9 or about 60 per cent yielded positive complement-fixation reactions with salt solution antigens of variolous and cowpox viruses. While the degree of complement absorption was relatively weak in all instances the reactions were generally stronger with the variolous antigens than with the cowpox antigens.

5. Alcoholic extracts of variolous and cowpox viruses possessed little or no antigenic sensitiveness.

6. These complement-fixation reactions have demonstrated the close biological relationship between the antibodies of vaccinia and variola; it is probable that complement-fixation reactions with salt solution antigens of the contents of smallpox lesions or fresh cowpox virus will prove of some value in the diagnosis of smallpox. Chang and Chen,[34] however, have observed but 10 per cent positive reactions with antigens of variolous lymph.

COMPLEMENT-FIXATION IN VARICELLA.

In 1916 I also applied the complement-fixation reaction in a study of varicella[35] with the following results:

1. Of 28 sera from patients who presented the first evidences of varicella in from four to forty-two days prior to the tests, 11 or 39

per cent reacted weakly positive with a salt solution antigen of varicella crusts.

2. Of 24 sera tested with a salt solution antigen of the contents of varicella vesicles, 7 or about 30 per cent reacted weakly positive.

3. Negative reactions with both varicella antigens were generally observed with the sera of adult persons who had varicella in childhood. In varicella the Wassermann reaction is negative in the absence of syphilis.

4. The sera of several syphilitics yielding strongly positive Wassermann reactions reacted negatively with the varicella antigens.

5. In all instances the degree of complement absorption with the sera of varicella patients and varicella antigens was slight (reactions weakly or doubtfully positive).

6. Cowpox and variola antibodies did not absorb complement with varicella antigens. The 4 weakly positive reactions observed with a salt solution extract of cowpox virus (calf) are to be ascribed to the presence of cowpox antibodies in the sera, similar results having been observed in the study of complement-fixations in vaccinia and variola.

7. While these experiments have shown that there is present in the sera of persons suffering with varicella an antibody in the nature of an amboceptor which will absorb complement in the presence of an antigen prepared of the cutaneous lesions of this disease, yet the percentage of positive reactions and particularly the degree of complement absorption is small; while immunity principles are in all probability present in the body fluids of persons for years after an attack of varicella these could not be detected by the complement-fixation tests in this study; all positive reactions were observed during or soon after an attack of the disease and at the time of probable highest concentration of antibodies. A more delicate technique would probably yield a higher percentage of positive reactions as is usual in all complement-fixation tests with bacterial antigens, but in this study this was avoided in order to guard against the possibility of non-specific absorption of complement.

Girard[36] has also observed positive reactions in varicella and regards the test of value in differentiating between chicken pox and smallpox.

COMPLEMENT-FIXATION IN OTHER DISEASES OF HUMAN BEINGS.

Acute Anterior Poliomyelitis.—Wollstein,[37] using extracts of various organs and cerebrospinal fluid as antigens, obtained uniformly negative results in a small series of cases. Lebredo and Recio[38] claim to have obtained a fixation reaction in 1 of 3 cases tested, using extracts of various organs as antigens, while Gay and Lucas[39] in a

series of complement-fixation tests found no evidence of antibodies or antigen in the spinal fluid and blood serum of poliomyelitic monkeys or human beings.

Complement-fixation tests conducted by Freese and myself[40] with the cerebrospinal fluids of 130 persons suffering with acute anterior poliomyelitis and salt solution extracts of various tissues yielded from 6 to 16 per cent weakly positive reactions with extracts of spinal cord, pons and medulla, cerebellum, cerebrum, and 15 to 17 per cent positive reactions with extracts of poliomyelitic liver and spleen. Control antigens of non-poliomyelitic human and rabbit liver yielded negative reactions, while extracts of spleen yielded from 5 to 7 per cent positive reactions. With alcoholic extracts of these tissues the tests were uniformly negative.

The cerebrospinal fluids of normal persons and children suffering with whooping cough and measles yielded negative reactions with all extracts. The cerebrospinal fluids of several paretics yielded strongly positive reactions with all salt solution and alcoholic extracts.

Complement-fixation tests with the sera of 58 persons in the various stages of acute anterior poliomyelitis yielded slightly positive reactions with 2 to 4 per cent of sera with salt solution extracts of spinal cord, pons and medulla, cerebellum and cerebrum. Tests with extracts of poliomyelitic liver and spleen were uniformly negative, as were all tests employing alcoholic extracts of these different tissues.

With polyvalent suspensions of diplococci and streptococci cultivated from persons suffering with acute anterior poliomyelitis, weakly positive reactions were observed with 6 to 9 per cent of cerebrospinal fluids of 35 persons suffering with the disease. With sera all tests were negative, as likewise all tests with fluids and sera employing suspensions of the diphtheroids and Gram-negative bacilli cultivated from poliomyelitis cases. These results indicate that the micrococci of poliomyelitis may produce antibodies during the course of the disease.

The Wassermann reaction in acute anterior poliomyelitis is uniformly negative with both cerebrospinal fluid and blood serum.

These tests indicate that suitable salt solution extracts of various tissues from fatal cases of acute anterior poliomyelitis may serve in a very small percentage of cases to fix or absorb a small amount of complement in the presence of large amounts of cerebrospinal fluid, but we considered the reactions too irregular and weak to be of any practical value in the diagnosis of the disease.

Neustaedter and Banzhaf,[41] however, have reported as high as 90.5 per cent positive reactions with spinal fluids and an antigen prepared by passing a 5 per cent water suspension of brain and cord of poliomyelitic monkeys through a Berkefeld or Heim filter, adding

trypsin to 1 to 50 dilution, digesting at room temperature for three hours and adding 0.4 per cent tricresol. Some positive reactions also occurred with syphilitic spinal fluids and sera as would be expected, but if these results are confirmed the complement-fixation test should be found a valuable diagnostic aid in this disease.

Much less work has been done along these lines in the serum diagnosis of *lethargic encephalitis;* Mutermilch and Nicolan[42] report negative results with the "koktoantigene" of Krans and Takaki.

Basedow's Disease.—Complement-fixation in this disease has commanded some attention although Galli-Valerio[43] and Miller and Fairband[44] have reported negative results. Berkeley[45] however, has recorded positive reactions with the sera of some individuals with toxic thyroid disease using an antigen prepared of normal thyroid glands of dogs prepared according to Koopman's method.[46] I have tried the antigen with the sera of 5 Wassermann negative individuals with well marked exophthalmic goiter but with negative reactions in all.

Malignant Tumors.—Despite a great deal of work devoted to complement-fixation in carcinoma and sarcoma and especially by von Dungern,[47] it may be stated that the occasional positive results that have been reported were due in part to accident or to some unknown and uncontrolled factor. I have used both the original alcoholic extract of malignant tumors of von Dungern and his later acetone extract of human blood corpuscles with an antiox hemolytic system and the three-hour room primary and secondary incubations, but with completely negative results in the case of sera from various clinical types of carcinoma in 11 non-syphilitic individuals. Coca[48] has had a similar experience and reviewed the literature. It is true that the serum-antigen mixtures sometimes gave retarded hemolysis as compared with the controls but this frequently happens in all complement-fixation tests because of the summation of anti-complementary effects of serum and antigen. The sera of non-cancerous syphilitic individuals frequently yielded positive reactions with both antigens. With the assistance of Miss Richter, I have also applied similar tests in a study of complement-fixation in Rous chicken sarcoma but with completely negative results.

Complement-fixation in Leprosy.—I have discussed in Chapter XL the subject of the Wassermann reaction in leprosy and here it may be briefly stated that numerous attempts have been made to develop a test specific for leprosy by using antigens of various extracts of leprous tissue, tubercle bacilli, tuberculin and other acid-fast bacilli since the lepra bacillus has never been successfully cultivated. But it is hardly profitable to review the literature which is covered by Lewis and Aronson,[49] who have also reported their own studies along these lines. Some positive reactions have been reported by various observers but none can claim a sufficient degree of specificity

or sensitiveness for practical purposes unless the work recently reported by Gomes[50] receives confirmation. He used *streptothrix leproides* defatted with olive oil and acetone according to Mejunkin's method and reports 50 to 95.23 per cent positive reactions in the various clinical types of leprosy.

Complement-fixation reactions also occur in *diphtheria* and especially with antigens of the diphtheria bacillus as observed by Moshage and myself[51] and they have been reported in *croupous pneumonia, meningococcus meningitis, Bacillus coli infections and plague.* They have also been used for studying the etiological relationship of various organisms to disease as, for example, that of *Bacillus acne* to skin lesions by Strickler, Schamberg and myself;[52] also in search of the unknown antigens of disease as, for example, for the organism of *Rocky Mountain fever* in the eggs of infected ticks by Davis and Petersen,[53] etc.

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CHAPTER XLVI.

SENSITIVENESS, SPECIFICITY AND PRACTICAL VALUE OF THE COMPLEMENT-FIXATION REACTION IN DISEASES OF THE LOWER ANIMALS.

The complement-fixation test has been also applied in the diagnosis of some of the infectious diseases of the lower animals with a great deal of success especially in dourine (trypanosomiasis), glanders and contagious abortion and its practical applications are being constantly widened.

COMPLEMENT-FIXATION IN TRYPANOSOMIASIS.

The technique of this test has been described in Chapter XXXIV and its sensitiveness, specificity and practical value discussed in Chapter XLIX. It has proven exceptionally valuable in the diagnosis of dourine or *Trypanosoma equiperdum* infections of horses and has been largely responsible for the eradication of the disease in the United States. It has also been the means of detection of the disease among animals being imported from trypanosome basins abroad and Kelser[1] has likewise found it valuable for the detection of animal carriers of surra in the Philippine Islands.

According to Mohler and Schoening[2] animals affected with other diseases existing in the United States do not react to the test for dourine.

Owing to the high cost of treatment infected horses are usually killed although as I have pointed out elsewhere[3] treatment with arsphenamin and its congeners should not be too expensive at the present time for selected animals. Rabbits infected with *Trypanosoma equiperdum* are readily cured and the blood reactions usually become negative about four to eight weeks thereafter.

COMPLEMENT-FIXATION IN TUBERCULOSIS OF CATTLE.

The complement-fixation test has not proven as sensitive as the tuberculin reaction for the detection of tuberculosis in cattle. With my method about 75 per cent positive reactions are observed among clinically tuberculous and tuberculin-positive cattle but the test has not proven as reliable or practical as in the diagnosis of tuberculosis of human beings although of distinct aid in the study of selected and valuable animals.

An extensive literature has accumulated upon the subject which it is hardly worth while reviewing although I wish to briefly summarize the studies made by Boerner and myself[4] with my new method and antigen.

Influence of Technical Factors.—The method of heating (inactivating) sera of the lower animals for the tuberculosis complement-fixation test was found to have considerable influence upon the reactions. Heating sera in a water-bath at 55° C. for fifteen minutes increased the percentage of non-specific reactions yielded by the sera of adult cattle, calves, sheep and horses. Heating the sera of these animals at 55° C. for thirty minutes increased still more the percentage of non-specific tuberculosis complement-fixation tests. Heating sera at 60° C. for thirty minutes greatly reduced the percentage of non-specific reactions and was found to be the method of choice for preparing the sera of cattle and other of the lower animals for complement-fixation tests.

The temperature and degree of the primary incubation had considerable influence upon the occurrence of non-specific and specific tuberculosis complement-fixation tests. A primary incubation of eighteen hours at 8° C. increased the amount of complement destruction, the amount of complement fixed by antigen alone and serum alone as well as the amount specifically fixed by mixtures of serum and antigen. The method of cold primary incubation of eighteen hours at 8° C. was found preferable to a water-bath incubation of one hour at 38° C. but required the use of smaller amounts of serum for avoiding non-specific reactions. With the technique employed in these tests as described in Chapter XXXII, sera used in dose of 0.025 cc. did not yield non-specific reactions while sufficient for yielding specific reactions.

The Occurrence of Tuberculosis Complement-fixing Substances in the Sera of Tuberculin-negative Cattle.—In the tuberculosis complement-fixation test with the sera of cattle, the occurrence of positive reactions with the sera of apparently healthy animals yielding negative tuberculin reactions proved very disturbing and materially reduced the practical diagnostic value of the reaction.

Porter,[5] Wyschelewsky[6] and others have reported positive reactions with the sera of apparently normal animals; Eichhorn and Blumberg[7] noted their occurrence with 45 or about 14 per cent of a series of 320 sera from animals without lesions and negative to the tuberculin test.

The nature and mechanism of these reactions have not attracted as much attention as their practical and theoretical importance demands. Eichhorn and Blumberg have suggested that the reactions in cattle may be due to the prior injection of tuberculin. They injected 2 healthy young cattle yielding negative complement-

fixation reactions with 2 cc. of tuberculin subcutaneously and found that the reactions became positive on the fourth day and strongly positive on the tenth day after injection. This condition persisted for four weeks followed by partial fixation and complete disappearance after six weeks.

In this connection it is rather significant that the sera of non-tuberculous calves almost invariably yield negative reactions. In one series of 60 sera Boerner and I did not observe a single positive reaction when the sera were heated at 60° C. for thirty minutes and tested with a warm primary incubation; in duplicate tests with these same sera employing cold primary incubation, positive reactions occurred in but 3 sera, and these were very weakly positive.

This suggests that the reactions with cattle sera are due to acquired tuberculous complement-fixing antibodies either as a result of small latent foci producing insufficient allergic sensitization for yielding tuberculin reactions and escaping detection at necropsy, or as a result of tuberculin injections, as suggested by Eichhorn and Blumberg. Hastings and Beach[8] however, have shown that various acid-fast bacilli other than tubercle bacilli may produce skin lesions in cattle capable of sensitizing them to tuberculin and the question arises whether or not complement-fixing antibodies are likewise produced and responsible for these reactions.

All of these reactions in our work occurred with the sera of cattle which had received 2 or more injections of tuberculin some time prior (four to twelve months) to the complement-fixation tests with the following results:

The sera of 6 to 15 per cent of tuberculin-negative cattle showed weakly positive but specific tuberculosis complement-fixation tests.

These reactions were not caused primarily by non-specific factors as result from heating sera at 55° C., temperature and duration of primary incubation, etc.

The substance in cattle sera responsible for them was usually removable in whole or in part by prolonged absorption of the sera with washed tubercle bacilli which indicated their specific amboceptor nature. These amboceptors were not usually present in the sera of calves.

The Diagnostic Value of the Complement-fixation Test.—Using the antigen and technique described in Chapter XXXII Boerner and I tested the sera of 162 clinically non-tuberculous and tuberculin-negative cattle and 68 tuberculous and tuberculin-positive animals with the following results:

Specific reactions due to tuberculin injections or clinically non-detectable lesions of tuberculosis were observed in 6 to 15 per cent.

Among dairy cows yielding positive tuberculin reactions, positive complement-fixation reactions occurred with 15 per cent of sera in

tests conducted with 0.05 cc. serum and warm incubation; with 0.025 cc. serum and refrigerator incubation, positive reactions occurred with 50 per cent of sera.

Among tuberculous cattle yielding positive tuberculin reactions and showing lesions of tuberculosis, positive reactions occurred with 50 per cent of sera in tests employing 0.05 cc. serum and a warm incubation; with 0.025 cc. of serum and refrigerator incubation positive reactions occurred with 72 per cent of sera.

The sensitiveness of the tuberculosis complement-fixation test was highest with freshly prepared antigens; little or no difference was noted with antigens of human and bovine bacilli.

Under these conditions we concluded that the complement-fixation reaction is not a practical diagnostic test for tuberculosis of cattle on a large scale. The fundamental reason for failure is an apparent lack of production of complement-fixing antibodies. Living tubercle bacilli engender a very high degree of allergic sensitization, but relatively small amounts of complement-fixing and other serum antibodies. However in the study of individual and especially valuable animals the test would appear to be well worth while along with tuberculin tests and clinical examinations.

COMPLEMENT-FIXATION IN GLANDERS.

In the diagnosis of glanders of horses and some of the other lower animals the complement-fixation test has rendered almost as great a service as has the Wassermann test for syphilis among human beings. It has proven particularly useful in the detection of the disease in horses and has been largely responsible for its successful eradication.

Dr. Fred Boerner and I in an extensive study of my method have found *the test very sensitive and specific for the detection of the disease in horses.* Positive reactions have occurred in less than a week after the onset of the disease. Unfortunately the normal sera of *mules* like those of rabbits and dogs, tends to give non-specific reactions as discussed in Chapter XXVIII although with the method described in Chapter XXXV mule sera can be successfully tested and positive reactions are of value in diagnosis along with agglutination and mallein tests.

The sera of normal horses do not give positive reactions although Boerner[9] has recorded positive reactions with the serum of a horse free of all clinical evidences, which did not receive injections of mallein and found in a stable in which glanders was not known to have been present.

As shown by Kelser and Hardenbergh[10] malleinization by intradermal injection frequently produces positive complement-fixation reactions; ophthalmic applications do not yield any at all. Positive reactions were demonstrated as early as twenty-four hours subse-

quent to injection and have persisted as long as thirty-two days although the average animal does not develop positive reactions until four to seven days after injection which persist approximately two weeks. Kelser and Hardenbergh state that when it is desired to supplement an intradermic mallein test with the complement-fixation test, the blood should be taken immediately after the forty-eight-hour reading of the former. After this time a period of approximately thirty days should be allowed before blood tests are made.

True carriers of *Bacillus mallei* are not supposed to exist but the complement-fixation test commonly detects the disease in animals showing no symptoms. When checked with mallein tests positive blood reactions are accepted as indisputable evidences of the disease.

COMPLEMENT-FIXATION IN BOVINE CONTAGIOUS ABORTION.

While the agglutination test is probably employed more widely than the complement-fixation test for the diagnosis of contagious abortion of cattle, yet as shown by Boerner and Stubbs[11] and confirmed in a study by Boerner and myself with the sera of 229 cattle tested by my method, the serum diagnosis of the disease is best served by conducting both tests.

In approximately 96 to 97 per cent of cases the two tests yielded similar reactions. From 2 to 3 per cent gave true complement-fixation and falsely negative agglutination reactions while approximately 1 per cent gave falsely negative complement-fixation and true agglutination reactions. It would appear therefore that the serum diagnosis of the disease is best served by conducting both tests and especially in the study of individual animals since the combination detects from 2 to 4 per cent reactors which would be classed as negative if either one of the tests alone were used.

The complement-fixation reactions are more decisive and leave a smaller number of doubtful reactions. The test is highly specific and normal cows or animals suffering with other diseases do not yield falsely positive reactions. It is also very sensitive and readily detects the disease when symptoms are absent or escape observation.

Of further interest in this connection is the possibility of *Bacillus abortus* infections of women and children and especially since Evans,[12] Feusier and Meyer[13] have shown the close serological relationship of the organism to *Bacillus melitensis* of Malta fever, but this subject is discussed with more detail in Chapter XLV.

COMPLEMENT-FIXATION IN EQUINE CONTAGIOUS ABORTION.

Since Meyer and Boerner[14] showed the etiological relationship of *Bacillus abortus equi* to contagious abortion of mares and the diagnostic value of agglutination and complement-fixation reactions in diagnosis, the latter tests have been widely and successfully

employed. The tests however, do not appear to be as decisive and specific as in the case of contagious abortion of cows although serviceable as aids in diagnosis.

COMPLEMENT-FIXATION IN OTHER DISEASES OF THE LOWER ANIMALS.

Various investigators have reported positive complement-fixation reactions in *hog cholera* with different antigens as Healy and Smith[15] employing an extract of mesenteric glands and King and Drake[16] with *Spirochæta hyos* but the results have not been confirmed.

Heller and Tomarkin,[17] Friedberger,[18] Baroni,[19] Berry and Mann[20] have applied it to the diagnosis of *rabies* with various antigens but the earlier results have not been confirmed and I am quite sure that the positive reactions observed with the sera of rabbits inoculated with fixed virus like those reported recently by Gorovits and Vlasova[21] have been due to the non-specific reactions sometimes observed with normal rabbit sera. Certainly I could obtain no true reactions with antigens of rabbit brain and spinal cord under proper technical conditions.

The complement-fixation reaction has failed in the diagnosis of *anthrax* and has not been used in the diagnosis of *symptomatic anthrax* (black leg). To the best of my knowledge it has not been used so far in the diagnosis of *bacillary white diarrhea* of fowls in which the agglutination test with antigens of *Bacillus pullorum* (*Salmonella pullorum*) is being so widely and successfully employed, but it would appear well worth while giving the test a trial as it is apt to be slightly more sensitive.

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CHAPTER XLVII.

SENSITIVENESS, SPECIFICITY AND PRACTICAL VALUE OF THE COMPLEMENT-FIXATION REACTION IN THE IDENTIFICATION OF BACTERIA AND TITRATION OF IMMUNE SERA.

THE COMPLEMENT-FIXATION REACTION IN THE DIFERENTIATION OF BACTERIA.

The complement-fixation reaction has proven very useful and somewhat more sensitive than the agglutination reaction for the differentiation of closely related bacteria although the technique is more complicated and the chances of error greater unless due precautions are taken in avoiding non-specific reactions if rabbits are employed for the preparation of the immune sera.

In conducting these tests each organism is injected into rabbits or some other suitable animal until large amounts of antibody are produced. Each immune serum is then tested in varying amounts with each bacterial antigen to determine the smallest amount of serum giving positive reactions. In this manner Amako and Kojima[1] have sought to classify the *dysentery group*, Williams, Raiziss and myself[2] the *typhoid-colon group*, etc.

Hooker[3] and Robinson[4] have also grouped *typhoid* bacilli into serological types by this method and I have shown[5] the close relationship of *Bacillus hofmanni* to the diphtheria bacillus by it.

I have likewise used the test for a study of *streptococci* and especially in scarlet fever.[6] Floyd and Wolbach[7] found it helpful in classifying streptococci and reported a marked degree of specificity between antigens of a given sugar group and corresponding antisera. Kinsella[8] has been able to group the streptococci of subacute endocarditis into three types; Swift and Kinsella[9] have also grouped the streptococci of acute rheumatic fever and found that non-hemolytic strains could be roughly grouped in three types bearing no relation to fermentation reactions. Howell[10] has found no serological relationship between hemolytic and non-hemolytic strains but observed some grouping according to disease or source. Hitchcock[11] found that non-hemolytic streptococci form an antigenically distinct but entirely heterogenous group impossible of differentiation into distinct types.

Gonococci, meningococci, influenza bacilli and other organisms have been also grouped by complement-fixation and agglutination

tests and in an unpublished work by Severac and myself with 21 strains of *staphylococci* from various sources, some evidences of grouping by complement-fixation and agglutination reactions were apparent which bore no relation to such biological reactions as pigment production, liquefaction of gelatin, coagulation of milk or fermentation of sugars.

Much less has been done with the *anërobes* but Starin and Dack[12] have recently stated that it is possible to divide *Bacillus botulinus* (*Clostridium botulinum*) into types A and B by means of the reaction.

THE COMPLEMENT-FIXATION REACTION FOR THE DETECTION OF BACTERIA AND TOXINS.

The complement-fixation reaction has also been used for the detection of typhoid bacilli in water by Rosler[13] and for the cholera bacillus in feces by Nidrigailoff[14] and Amaka and Kojima[15] as substitutes for bacteriological examinations. In these tests the water and feces were used as antigens along with highly potent anti-typhoid and anticholera sera but my own tests with artificially inoculated water, feces and urine were unsatisfactory and generally negative unless very large numbers of organisms were employed. Patterson has likewise found the test without value in the detection of gonococci in vaginal secretions of women with chronic gonorrhea.

Detection of Bacillus Botulinus and Toxin in Foods.—But Kelser[16] has applied the test with great success in the detection of *Bacillus botulinus* (*Clostridium botulinum*) in canned foodstuffs and gives detailed directions for the preparation of antigens. Starin and Dack have confirmed his work and found that the bacillus inoculated into peas, corn and spinach produced antigens for four different antisera, positive reactions occurring in some instances as early as sixty hours after inoculation and persisting for eight weeks.

COMPLEMENT-FIXATION IN THE STANDARDIZATION OF POLLEN EXTRACTS.

Clock[18] has advocated the complement-fixation test as a means for standardizing pollen extracts for desensitization in hay fever. It is not only more senstitive than nitrogen determinations but likewise highly specific; by it Armstrong and Harrison[19] have been able to distinguish giant and short ragweed pollen extracts from each other.

Miss Rule and I have succeeded in immunizing rabbits with ragweed pollens to the point where 0.5 cc. of 1 to 160 dilutions of serum (0.003 cc.) gave strongly positive reactions with pollen extracts used in one-third of their anticomplementary units.

But the practical value of the complement-fixation test in the standardization of ragweed pollen extracts has been questioned

by Armstrong and Harrison[20] who, in a recent report, have stated that "the complement-fixing property of ragweed pollen extracts is due to a labile fraction, is one of the first properties of the extract to deteriorate, and its rate of deterioration bears no definite relation to the rate of deterioration of the portion of the extract which causes shock.

"The complement-fixing property of ragweed pollen extract is a non-essential attribute insofar as the value of the extract for treatment is concerned.

"Complement-fixation as a means of standardization of ragweed pollen extracts should be applied only to extracts of stable antigenic nature, which, however, should be tested soon after extraction is completed.

"Complement-fixation for standardization of ragweed pollen extracts which are not stable in antigenic properties is fraught with danger, because overdosage may result; for some extracts which are relatively unstable as regards their complement-fixing properties may lose the latter, while their ability to cause shock is relatively unaltered."

COMPLEMENT-FIXATION IN THE STANDARDIZATION OF IMMUNE SERA.

The complement-fixation test has also been widely employed in the standardization of antistreptococcus, antigonococcus, antipneumococcus, antimeningococcus, antistaphylococcus and antianthrax sera employed in the treatment of disease.

Its value in this connection rests upon the assumption that the amount of complement-fixing antibody present is in ratio to the protection and curative activity of the serum even though the complement-fixing antibody itself may not be destructive for the organism.

In a broad and general manner this would appear to be correct to the extent that immune sera containing most complement-fixing antibody likewise contain most agglutinin and opsonin and are most protective to the lower animals inoculated with fatal doses of the homologous organism. But there are apparently some well marked exceptions and notably in syphilis and glanders; furthermore the converse is not true and has greatly reduced the practical value of the reaction as a means for the standardization of these sera, that is a serum may be very low in complement-fixing titer and yet prove, protective and curative in experimental injections. This is brought out in Table 81 summarizing the results of experiments by Miss Trist, Miss Yagle and myself with products kindly furnished by Dr. F. M. Huntoon and Mr. Roos:

TABLE 81.—THE RELATION BETWEEN COMPLEMENT–FIXATION, AGGLUTINATION AND PROTECTIVE POWER OF ANTIBACTERIAL SERA.

Sera.	Agglutination titers.	Complement-fixation titers.	Protection.*
Antipneumococcus I . .	1 to 80	No fixation	1,000 fatal doses
Antipneumococcus I . .	1 to 120	0.5 cc. of 1 to 20	10,000 fatal doses
Antipneumococcus I . .	1 to 60	No fixation	1,000 fatal doses
Antistreptococcus . . .	1 to 120	0.5 cc. of 1 to 100	100 fatal doses
Antistreptococcus . . .	1 to 300	0.5 cc. of 1 to 100	150 fatal doses
Antistaphylococcus . .	1 to 320	0.5 cc. of 1 to 200	
Antigonococcus . . .	1 to 40	0.5 cc. of 1 to 120	
Antimeningococcus . .	1 to 800	0.5 cc. of 1 to 320	
Antimeningococcus . .	1 to 400	0.5 cc. of 1 to 300	
Antimeningococcus . .	1 to 500	0.5 cc. of 1 to 150	

* Largest amounts of virulent twenty-four-hour broth culture against which mice were protected by 0.2 cc. of immune sera.

The antitoxic sera (diphtheria, tetanus and gas gangrene) are standardized by protection tests employing guinea-pigs; scarlet fever and erysipelas antisera are usually standardized by skin tests with susceptible human subjects; antipneumococcus serum is standardized by agglutination and by protective tests employing mice and antianthrax serum by protection tests employing mice and guinea-pigs while ordinary antistreptococcus, antimeningococcus and antistaphylococcus sera are usually standardized by agglutination tests. In these last three, however, I believe that the complement-fixation test could be used in addition with advantage.

Since Ramon[21] has established, on the principle of optimal proportions, a method for the titration of diphtheria toxin and antitoxin, interest has been renewed in the possibilities of the complement-fixation reaction for the same purpose since originally employed by Nicolle and Pozerski,[22] Armand-Delille,[23] Schürman and Sonntag[24] and others. Dean[25] has recently stated that by careful adjustment of all the factors which influence the reaction, complement-fixation can be demonstrated in mixtures of toxin and antitoxin but that the reactions are usually incomplete and too unsatisfactory for purposes of titration.

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CHAPTER XLVIII.

SENSITIVENESS, SPECIFICITY AND PRACTICAL VALUE OF THE COMPLEMENT-FIXATION REACTION IN MYCOTIC DISEASES.

The immunity of mycotic diseases has not received the attention that its importance merits. As a result surprisingly little attention has been given the subject of complement-fixation in ringworm, favus, actinomycosis and other mycotic diseases although what has been done indicates that complement-fixing antibodies are produced in the systemic infections like actinomycosis, sporotrichosis, sprue, and even some cases of superficial cutaneous infections like ringworm of the body and scalp. In the latter, general cutaneous phenomena like trichophytic erythema, lichen trichophyticum, etc., indicate that constitutional disturbances may occur and some investigators have stated that deep trichophytosis like kerion cutis, granuloma trichophyticum, sycosis parasitica, etc., are systemic diseases in which the fungi and their toxins may occur in the blood. These considerations are of importance in relation to serum diagnosis because the occurrence of antibodies in the blood may be expected to bear a relation to the severity and extent of the lesions.

COMPLEMENT-FIXATION IN ACTINOMYCOSIS.

Widal[1] as early as 1910 reported positive reactions in 8 cases of actinomycosis using the spores of cultures of pathogenic sporotricha as antigens since cultures of actinomyces were found unsuitable. Reactions also occurred in sporotrichosis and thrush, but these diseases could be differentiated clinically. More recently Nelson and Henrici[2] have reported positive complement-fixation reactions in the sera of 6 out of 8 actinomycotic cattle with four different antigens of *actinomycetes* isolated aërobically from cattle, and it would appear that the complement-fixation test is well worthy of trial in the serum diagnosis of actinomycosis occurring in cattle and human beings.

In my experience the antigens described in Chapter XXXIII may fix complement to some slight extent with the sera of syphilitic human beings containing large amounts of syphilis antibody and for this reason a control Wassermann test should be made with all human sera.

COMPLEMENT-FIXATION IN SPRUE.

Exceptionally encouraging results have been reported in sprue employing antigens of *Monilia psilosis*. Martinex[3] has reported positive reactions in moniliasis of the digestive tract and Michel[4] observed almost 100 per cent positive reactions with antigens of *Monilia psilosis*, negative reactions being observed in pellagra. Michel[5] has reported positive reactions in 81 cases, all showing the presence of *Monilia psilosis* in the feces.

COMPLEMENT-FIXATION IN SPOROTRICHOSIS AND BLASTOMYCOSIS.

Cases of transmission of sporotrichosis of horses to human beings have been reported, the slowly developing granulomata ending in degeneration and often mistaken for tuberculosis or syphilis. The disease is particularly prevalent among horses in the western states and the complement-fixation test would appear worthy of more extensive use as an aid in diagnosis.

Several strains of pathogenic sporotricha have been described but are grouped by Meyer and Aird as one species designated as *Sporothrix schenckii-beurmanni*. The possible successful application of the complement-fixation reaction in the diagnosis of human infections is indicated by the positive reactions occurring in a case reported by Moore and Davis[6] following a bite by a field mouse. Reactions occurred with four different antigens of strains of *Sporotrichum schenckii* including the one isolated from their case. Since the antigens may give cross complement-fixation reactions with syphilitic sera containing exceptionally large amounts of antibody it would appear advisable to conduct Wassermann tests with all human sera although these may be omitted in the case of sera from the lower animals. Very little work has been done with the complement-fixation reaction in blastomycosis but with generally negative results which is surprising in view of the close relationship of the disease to sporotrichosis.

COMPLEMENT-FIXATION IN RINGWORM.

Complement-fixation may occur in ringworm of the scalp and body with antigens of the trichophyta as probably first described by Strickler and myself.[7] We have found that, in the majority of cases of favus and ringworm of the scalp, complement-fixation occurs with fungus antigens, and while this test may have a limited practical value, owing to the ease with which the diagnosis of these diseases may be made from the clinical aspects, supplemented by a microscopic examination of diseased hairs, in the diagnosis of doubtful cases the complement-fixation test may prove of value.

Since ringworm of the scalp is to be regarded as a superficial invasion of the tissues of the scalp, the invasion of the fungus usually being limited to the shafts of hairs and the neighboring cells of hair follicles, it is of added interest that antibodies, which may be demonstrated in the serum of a person with this disease by the complement-fixation technique, may be produced by mere parasitic invasion without true infection, if the latter term is restricted to a process characterized by the passage of a microparasite through the barrier of epithelium to the tissues beneath. It is conceivable that the fungus of ringworm may produce soluble products which, being absorbed, are capable of stimulating the local and general body cells to produce antibodies.

Favus, on the other hand, is usually characterized by a well-marked invasion of all layers of the epiderm down to and not infrequently involving the underlying corium, so that in most instances the disease may be regarded as an infection in the true sense of the term.

Of interest in this connection is the demonstration by Plato, Truffi, Black and Massini of local and general allergic reactions in persons suffering with ringworm by the application or injection of "trichophytin," an extract prepared of cultures of the fungus indicating the presence of a lytic antibody in the body fluids of persons suffering with this disease.

Further interest in this subject refers to the possible interrelationship of the fungi of ringworm and favus as studied by a complement-fixation technique. Bodin has described cases in which lesions on the skin, similar to those produced by the trichophyton of ringworm, have been caused by a parasite of the nature of an achorion. Fox, Blaxall and Sabouraud have observed circinate lesions on the skin, and lesions like kerion on the scalp, similar to those produced by the trichophyta, which, on cultivation, gave a culture like that of favus. Such cases demonstrate the fact that the line of demarcation between the fungi of ringworm and favus is not well defined.

In our study complement-fixation with a polyvalent antigen of *microsporon* was found to occur in 78 per cent of persons suffering with ringworm of the scalp. The sera of 2 cases of favus studied yielded reactions with an antigen of the achorion of Schönlein.

The degree of reaction in both ringworm and favus was observed to depend in general, first, on the severity of the infection, and second, on the duration of the infection.

A culture of the scales of tinea versicolor, regarded as a doubtful culture of *Microsporon furfur*, reacted weakly or irregularly with the sera of ringworm and favus patients, and negatively with the serum of a case of tinea versicolor.

The ringworm and favus antibodies fixed complement best with their respective antigens, but with relatively large doses of serum

this specificity was not observed, owing probably to a biologic relationship of *Microsporon audouini* and *Achorion schönleinii*.

The antigens of the fungi did not fix complement with the sera of syphilitics and those of persons suffering from scabies, impetigo contagiosa, eczema, acne vulgaris and other diseases.

Greenbaum[8] has also reported about 30 per cent weakly positive reactions in a series of 25 cases of ringworm of the scalp and body. Cross complement-fixation reactions also occurred with antigens of *Microsporon audouinni* of favus, indicating the biological relationship of the organisms. In exceptional cases of extensive infections and for differentiating ringworm from inflammatory diseases closely resembling it in which mycelia are not found or their identity uncertain, the complement-fixation test would appear worthy of trial.

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CHAPTER XLIX.

SENSITIVENESS SPECIFICITY AND PRACTICAL VALUE OF THE COMPLEMENT-FIXATION REACTION IN THE DIAGNOSIS OF TRYPANOSOMIASIS, ECHINOCOCCUS AND OTHER PROTOZOAL AND METAZOAL DISEASES.

As a class the pathogenic protozoa and metazoa are actively antigenic in the production of complement-fixing antibodies and especially in the case of those which are capable of penetrating deeply into the tissues like the trypanosomes, *Tænia echinococcus* and the various liver and lung flukes of schistosomiasis or bilharziasis. When the parasites remain superficially as in the case of most of the intestinal infestments with *Tænia saginata*, *Ascaris lumbricoides*, uncinaria, etc., antibody production is much less although it occurs in some instances. On the whole, however, the field of protozoal and metazoal diseases offers a wide one for investigation in serum diagnosis especially by complement-fixation in the various clinical varieties of malaria, Leishmaniasis, amœbiasis, schistosomiasis, lambliasis, piroplasmosis, coccidiosis, trichinosis, intestinal helminthiasis, etc.

COMPLEMENT–FIXATION IN TRYPANOSOMIASIS.

The complement-fixation reaction has proven very valuable in the serum diagnosis of trypanosomiasis of horses, camels and other of the lower animals. Schoening[1] has recently stated that over 350,000 tests have been conducted in the Bureau of Animal Industry for *Trypanosoma equiperdum* infections of horses (dourine) with extremely good results and reports the successful use of the test in the detection of trypanosomiasis of camels being imported into the United States infected with a trypanosome resembling *Trypanosoma brucei*, Bessemans[2] using antigens prepared after the method of Mohler. Eichhorn and Buck[3] has also reported very reliable and specific reactions; Watson[4] and others have found the reaction of particular value in the diagnosis of latent infections. The reactions however, are of a *group* character; that is, the sera of horses suffering with dourine (*Trypanosoma equiperdum*) will fix complement with antigens of other trypanosomes as *Trypanosoma brucei*, *Trypanosoma equinum*, etc.

Rabbits, dogs and guinea-pigs experimentally infected with *Trypanosoma equiperdum*, *Trypanosoma brucei* and other pathogenic strains regularly develop complement-fixing antibodies as shown by McIntosh,[5] Woods and Morris[6] and others. In the experiments of Miss Rule and myself conducted as described in Chapter XXXIII, the sera of rabbits infected with *Trypanosoma equiperdum* regularly yielded strongly positive reactions about seven days after inoculation as shown in the following results with one animal of a large series:

Preliminary to infection	–	–	–	–	–
Two days after infection				–	–
Five days after infection	2			–	–
Seven days after infection	4	3	1	–	–
Fourteen days after infection	4	4	4	4	4

White rats inoculated with *Trypanosoma equiperdum* usually succumb in five to seven days and the heated sera generally yield negative reactions. But by giving substerilizing doses of neoarsphenamin like 0.012 gm. per kilogram, we have been able to maintain the infections for one to three weeks and over 90 per cent have developed positive reactions on or about the eighth day after intraperitoneal inoculation.

LaCorte[7] has recently reported 79.5 per cent positive reactions in a series of 200 cases of suspected Chagas' disease employing an antigen of spleen from a puppy well infected with *Trypanosoma cruzi*.

The percentages of positive reactions in the different clinical types were as follows:

Nervous system	100.0 per cent
Cardiac system	87.2 per cent
Thyroid lesions	79.5 per cent
Glandular system	75.0 per cent
Undetermined forms	25.0 per cent

Wassermann reactions conducted with the same sera yielded 17 per cent positive reactions. In 13 per cent both reactions were positive. Most of the patients yielding positive Wassermann reactions showed evident syphilitic lesions. The sera of 16 normal individuals, 2 with malaria and 3 with other diseases reacted negatively, indicating the high specificity of the reaction and confirming the reports of Guerreiro and Machado,[8] Villela and Bicalho[9] and Leao[10] on the high specificity and practical value of the complement-fixation reaction in the diagnosis of this trypanosome disease.

Trypanosomiasis is also of interest from the possibility of yielding positive Wassermann reactions. Browning and McKenzie[11] and more recently Landsteiner and Van der Scheer[12] state that rabbits inoculated with living or dead trypanosomes produce the Wassermann substance capable of yielding positive reactions with

various lipoidal tissue extracts employed as antigens. But this has not been my experience with rabbits and rats inoculated with *Trypanosoma equiperdum* and tested as described in Chapter XXXIV with my new cholesterolized and lecthinized alcoholic extract of beef heart when the sera were heated at 62° C. for one-half hour to avoid the non-specific reactions sometimes yielded by normal rabbit sera. The results observed with one rabbit are shown in Table 82 as an example of those observed in a series. Similar results were observed in white rats when infections with *Trypanosoma equiperdum* were held in sufficient check by substerilizing doses of neoarsphenamin to permit the animals to live about three weeks.

TABLE 82.—COMPLEMENT-FIXATION REACTIONS IN EXPERIMENTAL TRYPANOSOMIASIS.*

	C. L. antigen;† serum doses.							Trypanosome antigen; serum doses.						
	.025.	.012.	.006.	.003.	.0015.	.0008.	Serum control.	.025.	.012.	.006.	.003.	.0015.	.0008.	Serum control.
Before injection	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1 week	—	—	—	—	—	—	—	4‡	3	1	—	—	—	—
2 weeks	—	—	—	—	—	—	—	4	4	4	4	4	2	—
3 weeks	—	—	—	—	—	—	—	4	4	4	4	4	4	—
4 weeks	—	—	—	—	—	—	—	4	4	4	2	1	—	—

* Infected with *Trypanosoma equiperdum*.
† C. L. refers to the new cholesterolized and lecithinized antigen.
‡ — = negative; 1 = +; 2 = ++; 3 = +++; 4 = ++++.

Since heating sera at 62° C. did not destroy antibody for antigens of *Trypanosoma equiperdum* I believe it may be stated that the negative reactions with the syphilis antigen were not to be ascribed to destruction of Wassermann antibody. It may be that positive reactions could be elicited with other extracts and other complement-fixation methods but insofar as my method and antigen are concerned, I doubt their occurrence in view of the negative reactions observed in rabbits examined repeatedly over three to six weeks of time and presenting lesions of trypanosomiasis in the testicles and elsewhere as well as intermittent periods of trypanosomes in the peripheral blood.

COMPLEMENT-FIXATION IN ECHINOCOCCUS DISEASE.

One of the earliest applications of the phenomenon of complement-fixation was by Ghedini[13] in 1906 who found positive reactions in postoperative cases of echinococcus or hydatid disease of the liver. Apphatie and Lorenz,[14] however, were apparently first to apply the test in diagnosis and in 1908 reported positive reactions in all of 25 cases examined with negative reactions in 117 controls.

Since then a rather large literature has accumulated which was thoroughly reviewed in 1915 by Zapelloni[15] covering about 700 cases and showing approximately 95 per cent positive reactions, while controls including the sera of individuals with *Tænia saginata* and other infestments reacted negatively. Prado[16] has reported 92.3 to 100 per cent positive reactions in 65 infested human beings and in 90.7 per cent of 150 infested lower animals and considers the complement-fixation test superior to the skin test and differential leukocyte count in diagnosis.

As is to be expected the reports in literature vary greatly on the percentage of positive and negative reactions and in opinions upon the specificity and practical value of the test because of the great variations in technique employed and especially in relation to the antigen. Owing to the rarity of the disease among human beings in and around Philadelphia my own experience has been quite limited, but in 2 cases yielding positive reactions, cysts of the liver were found at operation. Unquestionably *syphilis antibody may give positive reactions with echinococcus antigen and for this reason the Wassermann test should be conducted with each serum submitted for the echinococcus test.* If the former yields a positive reaction it is likely that the latter will be also positive and for this reason *a positive report in the case of the echinococcus test should be made with great caution in syphilitic individuals.* During the past ten years I have tested more than 6 echinococcus antigens with the sera of syphilitics free of hydatid disease and all have yielded these cross reactions with sera containing *large* amounts of syphilis antibody. In testing the sera of sheep, cattle and other of the lower animals Wassermann tests are not ordinarily required although I believe it is advisable to include a syphilis antigen to control possible non-specific reactions.

Otherwise, however, the test appears to possess a high degree of specificity and falsely positive reactions apparently do not occur as result of infestments with *Tænia saginata, Ascaris lumbricoides,* etc. This specificity is also apparent in complement-fixation tests employing the sera of rabbits immunized with echinococcus antigen. For example the serum of a rabbit injected with echinococcus material (see Chapter XX) yielded the following reactions with various antigens employed in doses equivalent to one-quarter of their anti-complementary units:

	Complement-fixation reactions.			
Antigens.	0.025 cc.	0.0125 cc.	0.006 cc.	Control.
Echinococcus . .	++++	++++	++++	–
Tænia saginata . .	–	–	–	–
Ascaris lumbricoides	–	–	–	–

Positive echinococcus reactions with the sera of Wassermann-negative individuals usually means the presence of hydatid disease in the

liver, spleen or elsewhere; a negative reaction, however, does not exclude the disease since small and early lesions may not engender sufficient antibody for detection in the test. The echinococcus complement-fixation test possesses therefore, a higher positive than negative value and is always to be employed as an aid in diagnosis when acceptable antigen is available (see Chapter XVI).

COMPLEMENT-FIXATION IN BILHARZIOSIS.

Recent investigations have shown that the complement-fixation test may be employed in the serum diagnosis of fluke infestations of human beings and the lower animals.

Weinberg[17] and Paccanaro[18] were first to demonstrate the presence of specific antibody in the sera of sheep infested with *Filaria hepatica*. Servantie[19] has confirmed certain of these early observations, utilizing extracts of flukes as antigen, and in addition has obtained positive results in a human case infested with this parasite. Ando[20] has applied the test in the diagnosis of paragonimiasis of China and Japan. While infestation of sheep with *Filaria hepatica* is common, producing a condition known as sheep rot, it does not frequently infest human beings although cases have been reported by Allen, Manson-Bahr, Khouri and others.

Yoshimoto[21] in 1910 and Sueyasu[22] in 1916 reported positive complement-fixation reactions in infestations with *Schistosomum japonicum* and thought that the tests possessed diagnostic value. Murray[23] reported successful diagnostic reactions employing as antigen alcoholic extracts of the cercariæ of *Schistosomum hæmatobium* from the livers of snails. Fairley[24] failed to obtain satisfactory results with antigens of adult bilharzia worms but evolved a test for detecting *Schistosomum mansoni* and *Schistosomum hæmatobium* infestations in man and experimentally infested monkeys by extracting the bilharzia cercariæ located in the livers of the infected molluscan intermediaries (*Planorbis boissyi* and *Bullinus dybowski*) with absolute alcohol as described in Chapter XVI. Fairley[25] also reported positive complement-fixation reactions in a series of goats experimentally infected with *Schistosomum spindalis;* group reactions occurred with antigens of *Schistosomum indicum* and *Schistosomum hæmatobium* but outside of the mammalian bilharzia group the reactions were specific—sera from no other helminthic infestation yielding pseudopositive reactions. Hamilton and Williams[26] have also reported positive reactions. In a more recent paper Fairley[27] has confirmed his earlier observations on the lipoidal nature of the antigen extractable with absolute alcohol from the wet and dried livers of infested snails although as stated in Chapter XVI, LeBas[28] would identify the antigenic factor in *Bilharzia* antigen with the protein or allied constituents of the extracts.

I have had no experience with complement-fixation in these fluke infestations but the reports summarized above indicate that it is of practical value not only in the diagnosis of the disease in veterinary practice but especially in human beings as supplementary to examination of the feces for ova and the estimation of absolute and relative increase of eosinophiles associated with an hepatic syndrome suggestive of some helminthic involvement of that organ. It is also probable that similar aid may be given in the serum diagnosis of urinary and intestinal schistosomiasis or bilharziasis and pulmonary distomiasis or paragonimiasis as well as in chemotherapeutic experiments with infested sheep. Since the bilharzia antigens contain sufficient lipoids for the fixation of complement with syphilis antibody it is always advisable to conduct a Wassermann test with human sera and to exercise great caution in reporting positive bilharzia reactions in the case of Wassermann-positive individuals.

COMPLEMENT–FIXATION IN INTESTINAL HELMINTHIASIS.

As a general rule *complement-fixation reactions in intestinal helminthiasis are apt to be weak permitting neither species differentiation nor the elimination of pseudo-positive reactions with syphilitic sera.*

These results are apparently due to lack of absorption of helminthic antigen from the intestinal tract although in some infestations and notably those with *Bothriocephalus latus* sufficient antibody may be produced for yielding positive complement-fixation reactions of diagnostic value. Undoubtedly the worms contain antigenic substances since the immunization of rabbits with saline extracts have resulted in the production of complement-fixing antibodies, as shown by Meyer[29] and later by Thiele and Embleton,[30] who likewise obtained negative reactions with syphilitic sera and tapeworm extracts.

Ghedini[31] first demonstrated positive complement-fixation reactions with the sera of individuals suffering with *Ascaris duodenalis* and *Ascaris lumbricoides* using saline extracts of the parasites as antigen. Weinberg and Parvu[32] noted similar results in infestations of horses with *Sclerostoma, Tænia plicata, Tænia perfoliata* and *Ascaris,* only the results were not so specific. Trist, Heist and myself[33] obtained positive reactions in dogs and especially in the case of tapeworm infestations, to a lesser degree with the ascarides and to a slight extent with the whipworm. Owing to the fact that dog sera are capable of yielding non-specific reactions we were unable to place the test on a practical basis but felt quite sure that specific helminthic antibodies were produced and especially in tapeworm infestations.

Usami[34] employing alcoholic extracts of hookworm obtained positive results with almost all his cases of uncinariasis but pseudo-

positive findings were recorded in ascariasis. Violle and Le Saint-Rat[35] failed to demonstrate specific reactions with the sera of tapeworm infested individuals using the acetone-insoluble lipoids as antigens. Trumpp[36] has reported positive reactions in oxyuris infestations and more recently Jerlov[37] and Becker[38] have reported positive reactions of practical diagnostic value in bothriocephalus infestations.

In my experience alcoholic extracts of intestinal parasites are capable of yielding cross complement-fixation reactions with syphilitic sera and for this reason Wassermann reactions should be conducted with all sera submitted for the helminthic tests. Saline extracts of the parasites also give cross reactions but in a weaker degree and only when sera contain large amounts of syphilitic antibody.

I doubt if the test can be placed on a reliable diagnostic basis for infestations with *Ascaris lumbricoides, Oxyuris vermicularis* or the whipworms but in the case of *Tænia saginata, Bothriocephalus latus* and other tapeworms and of hookworms disease it would appear that enough antibody production may occur to make the tests worth while as an aid in diagnosis in those cases yielding negative Wassermann reactions.

COMPLEMENT-FIXATION IN OTHER PROTOZOAN AND METAZOAN DISEASES.

Some attempts have been made to develop a complement-fixation test for malaria but without success. If a method can be evolved for cultivating the parasites more successfully *in vitro* for the preparation of antigen, I predict success since there is considerable data indicating the production of antibodies in this disease. It is apparent that a reliable and sensitive serum test would be of great value in the diagnosis of latent and chronic infections and possibly as an aid in guiding treatment.

Because of the same difficulty in securing enough parasites for the preparation of antigens the complement-fixation test has not yet been applied in trichinosis, coccidiosis, ambliasis and piroplasmosis although it is quite probable that antibodies are produced in amœbiasis, trichinosis and similar diseases in which the parasites invade the tissues of the host.

Amebic Dysentery.—Schuckmann[39] has employed the test successfully in differentiating among amœbæ cultivated successfully *in vitro*, and Craig[40] has recently described a complement-fixation test for the diagnosis of infestments with *Endameba histolytica* although owing to technical difficulties it is still too early to reach a conclusion regarding its practical value.

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CHAPTER L.

SENSITIVENESS, SPECIFICITY AND PRACTICAL VALUE OF THE KOLMER COMPLEMENT-FIXATION REACTION FOR THE IDENTIFICATION OF BLOOD STAINS AND SERA.

In my experience the complement-fixation method described in Chapter XXXVI has proven more sensitive and specific and of greater practical value than the precipitin test for the identification and differentiation of blood stains. It is true that the technique requires more work than the latter but the reactions are more definite, more sensitive and easier to read, with the result that one may testify in medico-legal cases with greater confidence in the accuracy of the results.

SENSITIVENESS.

The greater sensitiveness of the complement-fixation test has been impressed upon me during the past six years by comparative tests with a large number of various immune sera prepared from time to time. Precipitin tests were conducted in the usual classical manner with 0.1 or 0.2 cc. of immune serum and 1 cc. of varying dilutions of crystal-clear solutions of antigens, the readings being made at the end of an hour. The complement-fixation tests were conducted as described in Chapter XXXVI with 2 to 10 units of immune serum and Table 83 of selected good, poor and indifferent sera shows the *highest dilutions of each antigen giving* positive reactions in both tests:

Table 83.

Immune serum.	Serum antigen.	Precipitation.	Complement-fixation.
Antihuman	Human	1 to 5000	1 to 15,000
Antihuman	Human	1 to 1000	1 to 5,000
Antihuman	Human	1 to 1000	1 to 300
Antihuman	Human	1 to 1000	1 to 2,400
Antihuman	Human	1 to 1200	1 to 4,800
Antihuman	Human	1 to 400	1 to 600
Antihuman	Human	1 to 6000	1 to 9,000
Antihuman	Human	1 to 800	1 to 1,000
Antihuman	Human	1 to 3000	1 to 4,500
Antihorse	Horse	1 to 1280	1 to 80,000
Antihorse	Horse	1 to 1000	1 to 9,000
Antihorse	Horse	1 to 1200	1 to 7,000
Antisheep	Sheep	1 to 2000	1 to 5,000
Antisheep	Sheep	1 to 1280	1 to 10,000
Antiox	Beef	1 to 1280	1 to 10,000
Antiox	Beef	1 to 2500	1 to 30,000
Antiox	Beef	1 to 2000	1 to 24,000
Antiox	Beef	1 to 1000	1 to 1,200
Antihog	Hog	1 to 3000	1 to 6,000
Antidog	Dog	1 to 1600	1 to 25,000
Antidog	Dog	1 to 500	1 to 16,000
Antidog	Dog	1 to 1000	1 to 600
Anticat	Cat	1 to 200	1 to 800
Antichicken	Chicken	1 to 7000	1 to 11,000

In practically every instance the complement-fixation reactions were more sensitive although the precipitation and fixation titers were roughly parallel.

But this greater sensitiveness on the part of the complement-fixation reaction is particularly brought out in practical tests for the identification of blood stains as shown in the following examples taken from medico-legal cases during the past six years. In each instance the undiluted antihuman serum in dose of 0.1 or 0.2 cc. contained sufficient precipitin to give strong reactions with 1 cc. of 1 to 1000 or higher solutions of dried human blood and the tests were conducted with 1 cc. amounts of the stock solutions of the stains; the complement-fixation tests were conducted with 2 to 10 units of each antihuman serum and varying amounts of stock solutions of the stains 1 to 2 to 1 to 16.

TABLE 84.

Stains.	Precipitation.	Complement-fixation.			
		1 to 2	1 to 4	1 to 8	1 to 16
On shirt	Negative	4*	4	1	—
On towel	Positive	4	4	3	1
On shirt	Negative	3	1	—	—
On handkerchief	Positive	4	4	4	4
On knife	Negative	3	1	—	—
On oil cloth	Positive	4	4	4	4
On ax	Negative	4	2	—	—
On plastered wall	Positive	4	4	4	4
On floor	Positive	4	1	—	—
On leather automobile seat	Negative	4	2	1	—

* 4 = ++++; 3 = +++; 2 = ++; 1 = +; — = negative.

In my experience the complement-fixation test has been particularly useful when the amount of material for testing has been very small as in the case of stains partially removed by washing, inspissated material in the joints of pocket knives, small spots on flooring, etc.

SPECIFICITY.

For all practical purposes the complement-fixation test is absolutely specific for the detection of human blood when the possibility of stains being from the chimpanzee, gorilla and orang-outang can be excluded, as is usually easily possible in practical work. This is particularly true insofar as the differentiation between human blood and the blood of the ordinary domestic animals in the United States is concerned as shown in Table 85. In this particular experiment some cross complement-fixation occurred between the cow and sheep but in additional experiments with other immune sera these reactions did not regularly occur.

TABLE 85.—THE SPECIFICITY OF THE COMPLEMENT-FIXATION REACTION IN THE IDENTIFICATION OF BLOOD STAINS.

Extract dried blood.	Immune serum.	Reactions with varying dilutions of extract.				
		1 to 2	1 to 4	1 to 8	1 to 16	1 to 32
Human	Antihuman	4*	4	4	4	3
Cow	Antihuman	—	—	—	—	—
Sheep	Antihuman	—	—	—	—	—
Horse	Antihuman	—	—	—	—	—
Hog	Antihuman	—	—	—	—	—
Dog	Antihuman	—	—	—	—	—
Chicken	Antihuman	—	—	—	—	—
Human	Anticow	—	—	—	—	—
Cow	Anticow	4	4	4	4	4
Sheep	Anticow	3	—	—	—	—
Horse	Anticow	—	—	—	—	—
Hog	Anticow	—	—	—	—	—
Dog	Anticow	—	—	—	—	—
Chicken	Anticow	—	—	—	—	—
Human	Antisheep	—	—	—	—	—
Cow	Antisheep	1	—	—	—	—
Sheep	Antisheep	4	4	4	4	4
Horse	Antisheep	—	—	—	—	—
Hog	Antisheep	—	—	—	—	—
Dog	Antisheep	—	—	—	—	—
Chicken	Antisheep	—	—	—	—	—
Human	Antihorse	—	—	—	—	—
Cow	Antihorse	—	—	—	—	—
Sheep	Antihorse	—	—	—	—	—
Horse	Antihorse	4	4	4	4	4
Hog	Antihorse	—	—	—	—	—
Dog	Antihorse	—	—	—	—	—
Chicken	Antihorse	—	—	—	—	—
Human	Antihog	—	—	—	—	—
Cow	Antihog	—	—	—	—	—
Sheep	Antihog	—	—	—	—	—
Horse	Antihog	—	—	—	—	—
Hog	Antihog	4	4	4	3	2
Dog	Antihog	—	—	—	—	—
Chicken	Antihog	—	—	—	—	—
Human	Antidog	—	—	—	—	—
Cow	Antidog	—	—	—	—	—
Sheep	Antidog	—	—	—	—	—
Horse	Antidog	—	—	—	—	—
Hog	Antidog	—	—	—	—	—
Dog	Antidog	4	4	4	4	4
Chicken	Antidog	—	—	—	—	—
Human	Antichicken	—	—	—	—	—
Cow	Antichicken	—	—	—	—	—
Sheep	Antichicken	—	—	—	—	—
Horse	Antichicken	—	—	—	—	—
Hog	Antichicken	—	—	—	—	—
Dog	Antichicken	—	—	—	—	—
Chicken	Antichicken	3	2	1	—	—

* 4 = ++++; 3 = +++; etc.

This high specificity is also shown in the results of an experiment in Table 86 in which each antigen was used in a constant dose of one-quarter of its anticomplementary unit with varying amounts of immune serum.

TABLE 86.—COMPARATIVE PRECIPITIN AND COMPLEMENT-FIXATION REACTIONS FOR IDENTIFICATION OF BLOOD STAINS, SERA AND MEAT.

	Precipitin reactions with.			Complement-fixation reactions with.					
Immune serum.	Human serum.	Sheep serum.	Dog serum.	Extract human blood.	Human serum.	Extract sheep blood.	Sheep serum.	Dog serum.	Extract dog meat.
Antihuman . .	1:50*	—	—	1:400†	1:400	—	—	—	—
Antisheep . .	—	1:500	—	—	—	1:300	1:300	—	—
Antisheep . . No. 2	—	1:1000	—	—	—	1:600	1:600	—	—
Antidog . . .	—	—	1:100	—	—	—	—	1:600	1:600
Antidog No. 2 .	—	—	1:100	—	—	—	—	1:600	1:600

* Highest dilution of antigen giving positive reaction with 0.1 cc. immune serum; — = negative reactions with 1 to 25 dilution of antigen.

† Smallest amount of immune serum (heated at 56° C. for fifteen minutes) yielding positive reaction with one-quarter anticomplementary dose of extract (antigen); — = negative reactions with 0.0125 cc. immune serum, the largest amount employed.

It is true that an antihuman serum may give complement-fixation with bloods from the higher apes and especially the chimpanzee but not with the bloods of the lower monkeys like the *Macacus rhesus* and *Circopithicus niger*.

Antihorse immune serum also reacts with the bloods of the mule and ass as is likewise true in precipitin reactions, but this does not create confusion in medico-legal tests for human blood.

Furthermore antihuman sera prepared by injecting rabbits with human serum or defibrinated blood may also react with other human albumins as has been found with the precipitation test, but this does not introduce error or confusion in the identification of stains shown by *chemical tests to contain hemoglobin* as observed in the following results with an antihuman serum and various human albumins:

TABLE 87.

		Complement-fixation.				
Extracts.	Precipitation.	1 to 2	1 to 4	1 to 8	1 to 16	1 to 32
Serum	+	4	4	4	4	4
Blood	+	4	4	4	4	4
Semen	+	2	—	—	—	—
Vaginal secretion . .	+	1	—	—	—	—
Albuminous urine (1 per cent)	+	2	1	—	—	—
Sputum	+	3	1	—	—	—
Muscle	+	4	2	—	—	—

It is possible that the group antibodies for some or all of these substances could be removed by absorption as Hektoen has removed from antisemen serum precipitin for human serum and as I have removed complement-fixation antibody for human serum by the same technique, but *this is not required if chemical tests show that the stain is of blood.*

Finally the complement-fixation test does not differentiate among the bloods of different human beings. I am frequently called upon for these tests to determine whether or not children contain Negro blood but is impossible to decide by either the precipitation or complement-fixation test. In one of my medico-legal cases the complement-fixation test showed the presence of human blood on the shirt of the defendant. His attorney admitted this but asked me on the witness stand if I could prove that the stains were not of the defendant's own blood. Upon receiving a negative reply, several witnesses testified to occurrence of frequent nose bleedings on the part of the defendant which the attorney stated created the possibility of the stains on the shirt being the defendant's own blood rather than of the victim, but the former was convicted of a heinous crime involving sexual depravity.

PRACTICAL VALUE.

In my experience the complement-fixation test has been much more satisfactory than the precipitation test for the identification and differentiation of blood stains and sera. Group reactions are easily avoided by proper quantitative proportions between antigen and antiserum and especially by using the former in varying amounts with a constant dose of 2 to 10 fixing units of the latter. Stains several years old give as good reactions as those perfectly fresh. Furthermore, extracts of stains from wood, leather and earth are usually more satisfactory in the complement-fixation than in the precipitation reaction, probably because variations in the pH of the solutions are less likely to affect the former.

The complement-fixation reaction has been particularly useful in my experience when only very small amounts of material were available for testing because of its greater sensitiveness. Furthermore, the reactions are usually so clear-cut and positive and the known positive and negative controls so satisfactory that one has in greater degree the necessary assurance and conviction of the correctness of the results so essential before testimony in medico-legal cases is justified.

CHAPTER LI.

SENSITIVENESS, SPECIFICITY AND PRACTICAL VALUE OF THE KOLMER COMPLEMENT-FIXATION REACTION FOR THE IDENTIFICATION OF SEMINAL STAINS.

In my experience the complement-fixation test has proven much more sensitive than the precipitin test for the identification of seminal stains on clothing or other material. It is sometimes requested in medico-legal cases and are of great value when spermatozoa cannot be identified by microscopic examination or as confirmatory evidence of their presence.

Human antiserum sera will give positive complement-fixation and precipitin reactions with human blood and also with vaginal secretions free of spermatozoa so that due care must be exercised in excluding the presence of these substances in the stains. Since blood stains are commonly present in cases of rape on children *it is much better to conduct the tests with an antisemen serum from which the group antibodies for blood and vaginal albumins have been previously removed* as described in Chapter XXXVII.

SENSITIVENESS.

The greater sensitiveness of the complement-fixation test has been brought out in comparative tests with three antisera prepared by the immunization of rabbits with human semen. In the precipitin tests varying dilutions of semen were tested with 0.2 cc. of immune sera and the readings made after one hour of contact at room temperature. The complement-fixation tests were conducted as described in Chapter XXXVII. Table 89 and the following results showing the highest dilutions of semen giving precipitation and the highest dilutions of antisera giving ++++ complement-fixation reactions with amounts of semen-antigens corresponding to one-third of their anticomplementary units, demonstrate the superior sensitiveness of the complement-fixation reaction:

TABLE 88.

Antisera.	Precipitation.	Complement-fixation.
1	1 to 150	0.5 cc. of 1 to 320
2	1 to 600	0.5 cc. of 1 to 1500
3	1 to 400	0.5 cc. of 1 to 1200

TABLE 89.—COMPLEMENT-FIXATION REACTIONS FOR THE IDENTIFICATION OF HUMAN SEMEN.

Antigens.*	Precipitin tests.		Rabbit immune serum No. 1.						Rabbit immune serum No. 2.					
	Serum I.	Serum II.	0.02	0.01	0.005	0.0025	0.0013	Serum control.	0.02	0.01	0.005	0.0025	0.0013	Serum control.
Fluid semen	−	−	++++	++++	++++	++++	++	−	++++	++++	++++	++	−	−
Fluid semen	−	−	++++	++++	++++	++	+	−	++++	++++	++	−	−	−
Seminal stain	−	−	++++	++++	++	−	−	−	++++	++	+	−	−	−
Seminal stain	−	−	++++	++++	++	+	−	−	++++	+++	+	−	−	−

* Each antigen employed in one-third anticomplementary unit.

But in practical tests with seminal stains on garments the greater decisiveness of the complement-fixation reaction is even more apparent and especially when partially washed stains are submitted for examination since the amounts of extractable semen albumins are greatly reduced. This has been my experience several times during the past six years in the results of precipitation and complement-fixation tests conducted with antisemen sera previously absorbed with human serum and vaginal secretions summarized as follows:

TABLE 90.

Material.	Precipitation.	Complement-fixation. 1 to 2	1 to 4	1 to 8	1 to 16
Stains on a top shirt	+	4	2	—	—
Stains on an undershirt	—	3	1	—	—
Stains on drawers (partly washed)	—	2	1	—	—
Stains on handkerchief	+	4	2	—	—
Stains on handkerchief	+	4	4	1	—

SPECIFICITY.

As previously stated human antisemen sera will react to some extent with human blood, human serum and human vaginal secretions. I have not observed reactions with human urine containing albumin or with human saliva but it is possible that some antisera may react with these substances if they contain large amounts of albumins and for this reason I always include controls on human saliva in medico-legal tests for the detection of human semen, as described in Chapter XXXVII. These cross complement-fixation and precipitation reactions are brought out in the results of the following tests with an antiserum prepared by the immunization of rabbits with human semen and antigens of human semen, blood, serum, vaginal secretions (from a child with gonococcus vaginitis free of spermatozoa), saliva and albuminous urine:

TABLE 91.

Antigens.	Precipitation.	Complement-fixation. 1 to 2	1 to 4	1 to 8	1 to 16
Semen	1 to 150	4	4	1	—
Blood	1 to 400	4	4	2	—
Serum	1 to 400	4	4	2	—
Vaginal secretions	1 to 50	3	—	—	—
Saliva	Negative (1 to 10)	—	—	—	—
Albuminous urine (0.2 gm. per 100 cc.)	Negative (1 to 10)	—	—	—	—

After absorbing this antiserum with human serum and vaginal secretions as described in Chapter XXXVII the results of precipitation and complement-fixation tests were as follows:

TABLE 92.

Antigens.	Precipitation.	Complement-fixation 1 to 2	1 to 4	1 to 8	1 to 16
Semen	1 to 50	4	1	—	—
Blood	Negative (1 to 10)	—	—	—	—
Serum	Negative (1 to 10)	—	—	—	—
Vaginal secretions	Negative (1 to 10)	—	—	—	—
Saliva	Negative (1 to 10)	—	—	—	—
Albuminous urine	Negative (1 to 10)	—	—	—	—

Antihuman sera prepared by injecting rabbits with human serum will also give cross precipitation and complement-fixation reactions with antigens of human semen and vaginal secretions free of spermatozoa as well as with urine and sputum containing albumins, as shown in the results of a test summarized in Table 93.

TABLE 93.

Antigens.	Precipitation	Complement-fixation. 1 to 2	1 to 4	1 to 8	1 to 16
Serum	1 to 1200	4	4	4	4
Blood	1 to 800	4	4	4	4
Semen	1 to 120	4	4	2	—
Vaginal secretion	1 to 80	4	1	—	—
Albuminous urine	0	2	—	—	—
Sputum	0	3	1	—	—

By absorbing the serum with semen and vaginal secretions the cross reactions were removed:

TABLE 94.

Antigens.	Precipitation.	Complement-fixation. 1 to 2	1 to 4	1 to 8	1 to 16
Serum	1 to 800	4	4	3	1
Blood	1 to 350	4	4	1	—
Semen	Negative (1 to 10)	—	—	—	—
Vaginal secretions	Negative (1 to 10)	—	—	—	—
Albuminous urine	0	1	—	—	—
Sputum	0	2	—	—	—

Therefore if material is found to contain spermatozoa upon microscopic examination one may demonstrate their human origin by tests conducted with an antihuman serum prepared by injecting rabbits with human serum or defibrinated blood because this antiserum will not react with the spermatozoa of the lower animals.

PRACTICAL VALUE.

It is possible therefore to render the complement-fixation and precipitation tests specific for human semen by absorbing powerful antisera with human serum and vaginal secretions free of spermatozoa. For the latter I have employed vaginal washings from children with copious exudates. Under these conditions and by including adequate controls, the complement-fixation reaction as described in Chapter XXXVII has been rendered a very sensitive test for human spermatozoa and especially in the case of old stains on garments when identification by microscopic examination is impossible or the results indefinite.

CHAPTER LII.

SENSITIVENESS, SPECIFICITY AND PRACTICAL VALUE OF THE KOLMER COMPLEMENT-FIXATION REACTION FOR THE DETECTION OF MEAT ADULTERATION.

While meat adulteration is apparently not nearly as frequent in the United States as abroad and especially the introduction of raw and cooked horse, dog and cat flesh into sausages, yet it is advisable to have available a sensitive and specific means for the detection of this fraud. While the precipitation test has been mostly employed, the complement-fixation test has been rendered more sensitive and in my experience has proven much more satisfactory. A large number of sufficiently potent immune sera are required but these are not difficult to prepare for the complement-fixation test by the immunization of rabbits with unheated serum-muscle and cooked serum-muscle mixtures described in Chapter XXXVII. Precipitin sera of sufficient potency are more difficult of preparation. The same methods are applicable for the species identification of eggs, bones and other albuminous substances.

SENSITIVENESS.

The superior sensitiveness of the complement-fixation test has been apparent in a large number of experiments summarized in Table 95. The precipitation tests were conducted with varying dilutions of extracts of the raw and cooked flesh of dogs, beef, sheep, horses and cats in 1 cc. amounts with 0.2 cc. of homologous immune sera, but the results were quite unsatisfactory. The complement-fixation tests were conducted with 0.5 cc. of varying dilutions of each immune serum heated at 62° C. for thirty minutes and a constant dose of the extracts corresponding to one-third of their anticomplementary units, with much more satisfactory results.

Immunization of rabbits with raw serum results in producing antibody for both serum and extracts of raw flesh but practically none for extracts of boiled flesh. Immunization with extracts of raw flesh produces more antibody for antigens of raw flesh than for raw serum and almost none for boiled serum or flesh; for this reason I advised in Chapter XXXVII immunization with mixtures of raw serum and raw extract or heated serum and cooked extract for the preparation of immune sera for these tests.

TABLE 95.—COMPARATIVE SENSITIVENESS OF PRECIPITIN AND COMPLEMENT-FIXATION REACTIONS.

Immunization with.	Precipitation with.*			Complement-fixation with.†		
	Serum.	Raw flesh.	Cooked flesh.	Serum.	Raw flesh.	Cooked flesh.
Dog serum	1 to 400	− (1 to 10)	− (1 to 10)	0.5 cc. (1 to 300)	0.5 cc. (1 to 100)	− (1 to 40)
Dog serum	1 to 400	− (1 to 10)	− (1 to 10)	0.5 cc. (1 to 500)	0.5 cc. (1 to 150)	− (1 to 40)
Dog serum	1 to 100	− (1 to 10)	− (1 to 10)	0.5 cc. (1 to 80)	0.5 cc. (1 to 40)	− (1 to 40)
Dog serum	1 to 500	0	0	0	0.5 cc. (1 to 300)	0
Dog serum	(1 to 500	0	0	0	0.5 cc. (1 to 320)	0
Raw dog flesh	− (1 to 10)	− (1 to 10)	− (1 to 10)	0.5 cc. (1 to 40)	0.5 cc. (1 to 100)	− (1 to 40)
Raw dog flesh	− (1 to 10)	− (1 to 10)	− (1 to 10)	0.5 cc. (1 to 40)	0.5 cc. (1 to 60)	− (1 to 40)
Raw dog flesh	0	− (1 to 10)	0	0	0.5 cc. (1 to 120)	0
Raw dog flesh	0	− (1 to 10)	0	0	0.5 cc. (1 to 100)	0
Cooked dog flesh	− (1 to 10)	− (1 to 10)	− (1 to 10)	− (1 to 40)	− (1 to 40)	0.5 cc. (1 to 100)
Cooked dog flesh	− (1 to 10)	− (1 to 10)	− (1 to 10)	− (1 to 40)	− (1 to 40)	0. 5cc. (1 to 50)
Cooked dog flesh	− (1 to 10)	− (1 to 10)	− (1 to 10)	− (1 to 40)	− (1 to 40)	0.5 cc. (1 to 50)
Cooked dog flesh	0	0	− (1 to 10)	0	0	0.5 cc. (1 to 100)
Cooked dog flesh	0	0	− (1 to 10)	0	0	0.5 cc. (1 to 80)
Cooked dog flesh	0	0	− (1 to 10)	0	0	0.5 cc. (1 to 160)
Raw beef flesh	0	− (1 to 10)	0	0	0.5 cc. (1 to 200)	0
Raw beef flesh	0	− (1 to 10)	0	0	0.5 cc. (1 to 180)	0
Raw beef flesh	− (1 to 10)	− (1 to 10)	0	− (1 to 40)	0.5 cc. (1 to 60)	0
Raw beef flesh	− (1 to 10)	− (1 to 10)	0	− (1 to 40)	0.5 cc. (1 to 100)	0
Cooked beef flesh	− (1 to 10)	− (1 to 10)	− (1 to 10)	− (1 to 40)	− (1 to 40)	0.5 cc. (1 to 100)
Raw mutton flesh	0	− (1 to 10)	0	0	0.5 cc. (1 to 300)	0
Raw mutton flesh	0	− (1 to 10)	0	0	0.5 cc. (1 to 280)	0
Raw mutton flesh	− (1 to 10)	− (1 to 10)	0	− (1 to 40)	0.5 cc. (1 to 80)	0
Raw mutton flesh	− (1 to 10)	− (1 to 10)	0	− (1 to 40)	0.5 cc. (1 to 100)	0
Cooked mutton	− (1 to 10)	− (1 to 10)	− (1 to 10)	− (1 to 40)	− (1 to 40)	0.5 cc. (1 to 60)
Raw horse flesh	− (1 to 10)	− (1 to 10)	− (1 to 10)	0.5 cc. (1 to 60)	0.5 cc. (1 to 200)	− (1 to 40)
Raw horse flesh	− (1 to 10)	− (1 to 10)	− (1 to 10)	0.5 cc. (1 to 100)	0.5 cc. (1 to 380)	0.5 cc. (1 to 50)
Cooked horse flesh	− (1 to 10)	− (1 to 10)	− (1 to 10)	− (1 to 40)	− (1 to 40)	0.5 cc. (1 to 120)
Raw Cat flesh	− (1 to 10)	− (1 to 10)	− (1 to 10)	0.5 cc. (1 to 50)	0.5 cc. (1 to 100)	− (1 to 40)
Cooked cat flesh	− (1 to 10)	− (1 to 10)	− (1 to 10)	− (1 to 40)	− (1 to 40)	0.5 cc. (1 to 80)

* Highest dilutions of extracts giving precipitation with 0.2 cc. of immune serum after contact at room temperature for one hour; − (1 to 10) means no visible precipitation with 1 cc. of 1 to 10 extract and 0.2 cc. of antiserum; 0 = not tested.

† Highest dilutions of immune sera in dose of 0.5 cc. giving ++++ reactions with one-third of the anticomplementary units of antigens; − (1 to 40) means none or incomplete fixation with 0.5 cc. of 1 to 40 dilution of antiserum; 0 = not tested.

Immunization of rabbits with heated serum or cooked meat extract results in much less antibody production but the antibody possesses a distinct species specificity for homologous cooked antigens even though it is commonly stated that the precipitin reactions are non-specific. My precipitin reactions with these cooked extracts and their antisera were too indefinite and unsatisfactory to warrant an expression of opinion but insofar as the complement-fixation test is concerned I have found the sera sufficiently sensitive and specific for the detection of cooked flesh in sausages. This is especially true if the adulteration is at least 1 to 4, that is, 1 part of dog or other adulterating flesh to 3 parts of beef or pork.

Practical tests were conducted by purchasing sausages in the market and adding dog, cat and other adulterations. In one of these experiments 1 ounce of minced dog and 1 ounce of minced cat flesh were added to 3 ounces of sausage stated to be of beef and pork. An extract was prepared as described in Chapter XXXVII, titrated and used in one-half, one-quarter, one-eighth, one-sixteenth and one-thirty-second of the anticomplementary unit with 2 units of antidog, anticat, antihog, and antibeef sera prepared by the immunization of rabbits with unheated serum-extract mixtures; the results were as follows:

TABLE 96.

Complement-fixation reactions.

Antisera.	1 to 2	1 to 4	1 to 8	1 to 16	1 to 32
Antidog	4	3	2	1	—
Anticat	4	2	—	—	—
Antibeef	4	2	—	—	—
Antihog	2	1	—	—	—

A second mixture was prepared by adding boiled cat and dog to the raw sausage in the same proportions and an extract prepared and tested with the results showing that the presence of thoroughly boiled dog and cat flesh could not be detected with these antisera:

TABLE 97.

Complement-fixation reactions.

	1 to 2	1 to 4	1 to 8	1 to 16	1 to 32
Antidog	—	—	—	—	—
Anticat	—	—	—	—	—
Antibeef	4	2	—	—	—
Antihog	3	1	—	—	—

But when the mixture was tested with antisera prepared by the immunization of rabbits with heated serum-extract mixtures slight but definite degrees of complement-fixation were observed:

TABLE 98.

Complement-fixation reactions.

Antisera.	1 to 2	1 to 4	1 to 8	1 to 16	1 to 32
Antidog	2	1	—	—	—
Anticat	2	—	—	—	—

The question of whether the complement-fixation test can detect traces of adulteration is one entirely of the potency of the immune sera employed. When adulteration is one-half to one-quarter, detection is quite an easy matter with ordinary immune sera. With an antisheep serum I have been able to detect the presence of 1 part of minced mutton in 20 parts of minced beef but it should be possible to detect much smaller amounts.

SPECIFICITY.

In my experience the complement-fixation test described in Chapter XXXVII is sufficiently specific for the detection of the raw flesh of the horse, dog and cat in sausages, "doggies," bolognas, etc. An antihorse serum, however, will react to the presence of mule flesh but this amounts to about the same thing insofar as detection of adulteration is concerned. The detection of thoroughly cooked horse, dog and cat flesh is less satisfactory because of the greater difficulty in preparing sufficiently potent antisera, but it is possible; in my experience boiling does not completely destroy the antigenic specificity of sera and meat extracts, as it is stated to do in relation to the production of precipitins. However, boiling does reduce the antigenic sensitiveness of these substances so that it is more difficult to prepare antisera but the antibodies produced possess a high degree of specificity for their cooked homologous antigens. While my tests were conducted with horse, dog and cat yet I believe the results are equally applicable to the detection of adulteration with human, goat, deer and the flesh of other animals sufficiently unrelated to the rabbit to make possible the preparation of potent immune sera. I have never tried to detect rabbit flesh but doubtless this is possible with antisera prepared by the immunization of guinea-pigs or goats (preferred) with rabbit serum-muscle mixtures.

PRACTICAL VALUE.

As previously stated the precipitin and complement-fixation tests have had a much wider application in Europe for the detection of meat adulteration than in the United States where this fraud is apparently much less commonly practised. But it is possible that a wider use of the tests in this country would detect adulteration more commonly than supposed and especially in the case of the cheap preserved meats like the popular "hot doggies" and bolognas.

On one occasion Miss Rule and I purchased bolognas, "doggies" and sausages of the cheaper grades in Philadelphia and tested extracts of each as described in Chapter XXXVII with antihuman, antihorse, antisheep, antibeef, anticat, antidog and antihog sera prepared by the immunization of rabbits with the raw or unheated sera of these animals.

All reacted negatively with the antihuman, antihorse and antidog sera but one of the sausages reacted with anticat serum as follows:

TABLE 99.

Extracts.	Complement-fixation reactions. 1 to 2	1 to 4	1 to 8	1 to 16	1 to 32	1 to 64
Bologna No. 1	—	—	—	—	—	—
Bologna No. 2	—	—	—	—	—	—
Bologna No. 3	—	—	—	—	—	—
"Doggie" No. 1	—	—	—	—	—	—
"Doggie" No. 2	—	—	—	—	—	—
"Doggie" No. 3	—	—	—	—	—	—
Sausage No. 1	—	—	—	—	—	—
Sausage No. 2	—	—	—	—	—	—
Sausage No. 3	3	1	—	—	—	—

Two of the three sausages reacted with antisheep serum indicating the presence of mutton and two of the three sausages reacted with antihog serum showing the presence of pork, which was of considerable interest in view of the fact that one showing the presence of pork was purchased in a Jewish meat shop. All reacted to beef, showing that this meat constituted the bulk of those examined, the results with antibeef serum being as shown in Table 100:

TABLE 100.

Extracts.	Complement-fixation reactions. 1 to 2	1 to 4	1 to 8	1 to 16	1 to 32	1 to 64
Bologna No. 1	3	2	1	—	—	—
Bologna No. 2	4	3	2	—	—	—
Bologna No. 3	3	2	1	—	—	—
"Doggie" No. 1	4	4	4	4	3	2
"Doggie" No. 2	4	4	4	2	1	—
"Doggie" No. 3	4	4	4	4	2	1
Sausage No. 1	3	2	1	—	—	—
Sausage No. 2	4	4	4	4	4	3
Sausage No. 3	2	4	4	3	1	—

Smoking does not appear to interfere with the reactions and salted meats are likewise successfully handled.

Of course it is not possible in tests of this kind to differentiate among the organs of the lower animals. For example a sausage containing beef spleen or lungs reacts with an antiserum prepared by injecting rabbits with beef serum-muscle mixtures. It is possible to prepare immune sera by injecting extracts of thoroughly washed organs which react best with antigens of these organs, but such sera are not required in practical tests for meat adulteration. The ordinary immune sera are species but not organ specific.

While I have not applied the technique described in Chapter XXXVII for the species identification of eggs, bones and other organic matter, yet it is highly probable that the complement-fixation test would prove equally satisfactory for these purposes.

CHAPTER LIII.

SENSITIVENESS, SPECIFICITY AND PRACTICAL VALUE OF THE KOLMER COMPLEMENT-FIXATION REACTION FOR THE DETECTION OF MILK ADULTERATION.

The complement-fixation test is much superior to the precipitin test for the identification of milk. In my experience the latter has been frequently very indefinite and unsatisfactory in tests conducted with 1 cc. amounts of varying dilutions of milk and 0.2 cc. of antiserum or 1 cc. of serum plus 2 or 3 drops of filtered fat-free milk. Possibly more extensive immunization of rabbits with milk would have resulted in better success but in my experience rabbits do not stand the injections well and while 3 to 6 injections usually produce large amounts of complement-fixing antibody only small amounts of precipitin are demonstrable.

SENSITIVENESS.

This superior sensitiveness is shown in Table 102 and in the following results of precipitation and complement-fixation tests with various antisera, the former showing the highest dilutions of fat-free milk reacting with 0.2 cc. of immune serum after contact of one hour at room temperature and the latter the highest dilutions of antiserum in dose of 0.5 cc. with one-third of the anticomplementary units of homologous milk antigens:

Table 101.

Antisera.	Precipitation.	Complement-fixation.
Anticow	1 to 160	0.5 cc. of 1 to 300
Anticow	1 to 1280	0.5 cc. of 1 to 3000
Anticow	1 to 100	0.5 cc. of 1 to 200
Anticow	1 to 50	0.5 cc. of 1 to 80
Anticow	1 to 60	0.5 cc. of 1 to 200
Antihuman	Negative (1 to 50)	0.5 cc. of 1 to 200
Antihuman	1 to 60	0.5 cc. of 1 to 300

Doubtless a goodly part of the unsatisfactory results with the precipitin tests is due to difficulties in making the readings because of the cloudiness of the milk antigens and it is probable that tests conducted with solutions of isolated and purified proteins would yield more sensitive reactions.

TABLE 102.—PRECIPITIN AND COMPLEMENT-FIXING ANTIBODY PRODUCTION DURING IMMUNIZATION WITH HUMAN AND COW MILK.

Antigen.	Route.	Amount.	Precipitin reactions after.								Complement-fixation reactions after.						
			Intervals.	Preliminary.	1 injection.	2 injections.	3 injections.	4 injections.	5 injections.	6 injections.	Preliminary.	1 injection.	2 injections.	3 injections.	4 injections.	5 injections.	6 injections.
Cow's milk	Intraperitoneal	2, 4, 6, 8 cc.	5 days	—*	—	—	—	—	D	..	—	—	0.05†	0.005	0.0025	D	
Cow's milk	Intraperitoneal	2, 4, 6, cc.	5 days	—	—	—	—	D	..	..	—	—	0.01	0.005	D		
Cow's milk	Intravenous	2, 4, 6, 6 cc.	5 days	—	—	—	—	—	—	D	—	—	—	0.01	0.005	0.002	D
Human milk	Intravenous	2, 4, 6, 6 cc.	5 days	—	—	—	—	—	—	—	—	—	—	—	0.02	0.01	D
Human milk	Intraperitoneal	2, 4, 6, 8,cc.	5 days	—	—	—	—	—	—	—	—	—	0.02	0.02	0.01	0.0025	0.001

* Negative reactions with 0.1 cc. serum and 1 cc. of 1 to 100 dilutions of milk or in tests using 1 cc. serum plus 2 or 3 drops of filtered milk.
† Smallest amounts of sera yielding positive reactions.

SPECIFICITY.

Curiously anticow sera may sometimes give weak complement-fixation reactions with antigens of human milk and the reverse as shown in Table 103; it is easy to avoid them by employing only high dilutions of immune serum but their occurrence is of considerable academic interest in relation to the possibility of a common antigen in milks of different species and worthy of further investigation.

TABLE 103.—THE NON-SPECIFICITY OF ANTISERA FOR HUMAN AND COW MILK.

	Fixation reactions with human milk.*				Fixation reactions with cow milk.*			
Lactosera.	0.025†	0.0125	0.006	0.003	0.025†	0.0125	0.006	0.003
Anticow	++	−	−	−	++++	++++	++	+
Anticow	−	−	−	−	+++	+	−	−
Anticow	−	−	−	−	++++	++	−	−
Antihuman	++	−	−	−	−	−	−	−
Anithuman	++++	++++	+	−	++	−	−	−
Antihuman	++	+	−	−	−	−	−	−

* Antigens used in one-quarter anticomplementary units.
† Varying amounts of immune sera.

Furthermore, by absorbing the antisera it is usually possible to remove these cross complement-fixing antibodies as shown in Table 104 and previously described in Chapter XXXVII. By these methods it is possible to render the complement-fixation test specific for human milk or cow's milk.

TABLE 104.—THE SPECIFICITY OF ABSORBED ANTISERA FOR HUMAN AND COW MILK

	Fixation tests with human milk.*				Fixation tests with cow milk.*			
Lactosera.	0.025†	0.0125	0.003	0.003	0.025†	0.0125	0.006	0.003
Anticow	−	−	−	−	++++	+	−	−
Anticow	−	−	−	−	++	−	−	−
Anticow	−	−	−	−	+++	+	−	−
Antihuman	+	−	−	−	−	−	−	−
Antihuman	++++	++	−	−	−	−	−	−
Antihuman	+	−	−	−	−	−	−	−

* Antigens used in one-quarter anticomplementary units.
† Varying amounts of immune sera.

But as is well known with the precipitation reaction, anticow serum also reacts with goat's milk and the reverse and the same is true of the complement-fixation reaction. It is true that the reactions are more sensitive with homologous milk antigens as shown in Table 105 but the differences are hardly sufficient for rendering either the precipitation or complement-fixation tests sufficiently specific for the medico-legal detection of adulteration of goat's

milk with cow's milk or the reverse. Furthermore, the use of the purified proteins have not improved the situation and absorption of anticow serum with goat's milk or antigoat serum with cow's milk removes so much antibody, as shown in Table 106, that the absorbed sera are too insensitive for practical use.

TABLE 105.—CROSS COMPLEMENT-FIXATION REACTIONS WITH ANTICOW AND ANTIGOAT LACTOSERA.

	Fixation reactions with cow milk.				Fixation reactions with goat milk.			
Lactosera.	1 to 2*	1 to 4	1 to 8	1 to 16	1 to 2*	1 to 4	1 to 8	1 to 16
Anticow	++++	++++	++	−	++	+	−	−
Anticow	++++	+++	−	−	++	−	−	−
Anticow	++++	++++	++++	+	++++	++	−	−
Antigoat	++++	++	−	−	++++	+++	+	−
Antigoat	++	−	−	−	+++	+	−	−
Antigoat	+++	+	−	−	++++	++	−	−

* Results with one-half, one-quarter, one-eighth and one-sixteenth of the anticomplementary units of respective antigens as described in Chapter XXXVI.

TABLE 106.—COMPLEMENT-FIXATION REACTIONS WITH ABSORBED ANTICOW AND ANTIGOAT LACTOSERA.

	Fixation tests with cow milk.				Fixation tests with goat milk.			
Lactosera.	1:2*	1:4	1:8	1:16	1:2*	1:4	1:8	1:16
Anticow before absorption with goat's milk	++++	++++	++	−	++	+	−	−
Anticow after absorption with goat's milk	++	−	−	−	+	−	−	−
Antigoat before absorption with cow's milk	++++	++	−	−	++++	+++	+	−
Antigoat after absorption with cow's milk	+	−	−	−	+	−	−	−

* Results with one-half, one-quarter, one-eighth and one-sixteenth of the anticomplementary units of respective milk antigens.

As expected anticow lactosera also give precipitation and complement-fixation reactions with serum and antihuman lactosera react with human sera; it is true, however, that the reactions are more sensitive with the respective milk antigens as shown in Table 107.

TABLE 107. CROSS REACTIONS WITH LACTOSERA AND SERUM ANTIGENS.

Lactosera.	Antigens.	Precipitation.	Complement-fixation.
Anticow	Cow's milk	1 to 1280	0.5 cc. of 1 to 3000
Anticow	Cow's serum	1 to 160	0.5 cc. of 1 to 300
Antihuman	Human milk	1 to 120	0.5 cc. of 1 to 300
Antihuman	Human serum	1 to 10	0.5 cc. of 1 to 40

Antihuman serum prepared by the immunization of rabbits with human serum may also give precipitation and complement-fixation

reactions with antigens of human milk but not nearly to the same degree as sera prepared by immunizing rabbits with human milk. Therefore if it is desired to test for human milk it is far better to use a known human lactoserum. The same is true in the case of cow's milk.

PRACTICAL VALUE.

It is readily possible, therefore, to render the complement-fixation test sufficiently sensitive and specific for differentiating between human and cow milk and in view of the increasing frequency with which human milk is being produced and purchased for the feeding of infants this becomes a matter of practical importance from the standpoint of detection of adulteration with cow's milk. Unfortunately it does not serve for the detection of adulteration of goat's milk with cow's milk which is sometimes suspected in those localities where goat milk is produced on a commercial scale.

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